Oxysterol Signature as Putative Biomarker in Niemann-Pick Type C and Inflammatory Bowel Diseases

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À mon père, Uli

À Jean-Nicolas

Et à tous ce qui ont cru en moi

SUMMARY

Cholesterol oxidation products, also named “oxysterols”, were first mentioned and studied in 1913 by I. Lifschütz while developing the worldwide famous products Eucerin® and Nivea® cream. He described oxysterols of non-enzymatic origin, being principally oxygenated at the sterol ring. In contrary enzymatically derived oxysterols were discovered 50 years later and appeared to be mainly side chain oxygenated sterols. However, a few oxysterols of both origins exist. Nowadays, it is known that oxysterols tightly regulate cholesterol homeostasis that plays a major role in human health. This regulation takes place by the mean of four different pathways. The first is the inhibition of the commonly activated sterol regulatory element binding protein (SREBP) pathway and the second is the activation of liver X receptors α and β (LXRα and LXRβ). The third action of oxysterols is the accelerated degradation of the 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR), a key enzyme of cholesterol synthesis. The final pathway triggered by oxysterols is the enhanced cholesterol esterification for cell storage. These regulatory pathways as well as other oxysterol mediated mechanisms that do not regulate cholesterol levels, appeared in the last years to be important for several human physiological processes. For example it was found that 25-hydroxycholesterol (25-OHC) possesses anti-viral functions. This illustrates that the physiological importance of oxysterols was underestimated and that their implications are still not completely unravelled. Oxysterol analysis, however, is tricky and hampered by several difficulties, such as the large excess of cholesterol, the low endogenous concentrations of oxysterols, the possible autoxidation of cholesterol and oxysterols, the cellular localisation of oxysterols in membranes, the possible modifications of oxysterols like esterifications, reductions, or sulphations and finally their chemical structures and properties making mass spectrometry (MS) analysis difficult and not very specific.

In this work, two liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) methods were established for the analysis of oxysterols in human diseases. The first method was developed and implemented for the analysis of oxysterols of non-enzymatic origin in a clinical setting of Niemann-Pick type C (NP-C) diagnosis. In addition, a second method was optimised for the analysis of enzymatically-derived oxysterols in the plasma of patients with Inflammatory Bowel Disease (IBD), opening a new research domain.

In the first part dealing with the analysis of NP-C-specific oxysterols, we were able to precisely, accurately, and robustly measure Cholestane-3β,5α,6β-triol (C-triol) and 7-Ketocholesterol (7-KC) following a Steglich esterification. We established reference ranges for NP-C diagnostics, which turned out to be neither age nor gender-dependent and we established the pre-analytical stability of the compounds. We found an important intra-individual variation for both analytes, but could show increased C-triol levels in all Niemann-Pick patients including Niemann Pick type A and B patients. Our results suggest that especially the C-triol is a biomarker for all three Niemann-Pick diseases.

In the second part dealing with the oxysterol fingerprint in IBD, we were able to separate in one chromatographic run 24(S)-hydroxycholesterol (24(S)-OHC), 25-hydroxycholesterol (25-OHC), 27-hydroxycholesterol (27-OHC), 7α,24(S)-dihydroxycholesterol (7α,24(S)-OHC), 7α,25-dihydroxycholesterol (7α,25-OHC), 7α,27-dihydroxycholesterol (7α,27-OHC), 7β,25-dihydroxycholesterol (7β,25-OHC), and 7β,27-dihydroxycholesterol (7β,27-OHC). The most important finding of our investigation in IBD is that 27-OHC and eventually 25-OHC are reduced in plasma of IBD patients compared to healthy volunteers. Both oxysterols are agonists for the LXR receptors, which are implicated in inflammation and thus may possibly participate in inflammatory mechanisms in IBD. The analysis of mono-and dihydroxycholesterols as biomarkers for IBD is a novel approach which shows promise but more IBD samples are required to corroborate the observed differences in oxysterol levels between the different groups, meaning the control group, patients with active Crohn’s disease or in remission, and patients with active Ulcerative colitis or in remission.

ZUSAMMENFASSUNG

Cholesterinoxidationsprodukte, auch "Oxysterole" genannt, wurden erstmals 1913 von I. Lifschütz erwähnt und untersucht, während er die weltweit bekannten Produkte Eucerin® und Nivea® Creme entwickelte. Er beschrieb Oxysterole, die nicht-enzymatisch gebildet werden und vor allem am Sterol-Ring oxidiert sind. Enzymatisch gebildete Oxysterole, die im wesentlich an den Seitenketten oxygeniert sind, wurden dagegen erst 50 Jahre später entdeckt. In wenigen Fällen werden Oxysterole durch eine Kombination dieser beiden Stoffwechselwege generiert. Heutzutage ist bekannt, dass Oxysterole die Cholesterinhomöostase kontrollieren, die ein wichtiger biochemischer Prozess darstellt und wesentlich auf die menschliche Gesundheit einwirkt. Die Regulierung der Cholesterinhomöostase wird durch vier verschiedene Mechanismen bedingt. Der erste Regulationsmechanismus ist die Hemmung der Cholesterinbiosynthese durch die Hemmung des *Sterol Regulatory Element-Binding Proteins* (SREBPs), während als zweiter Regulationsmechanismus, die Aktivierung der *Leber-X-Rezeptoren* α und β (LXRα and LXRβ) zu vermehrtem Cholesterinexport führt. Der beschleunigte Abbau der *3-hydroxy-3-methyl-glutaryl- coenzyme A reductase* (HMGCR), die als Schlüsselenzym der Cholesterinbiosynthese fungiert, ist der dritte Regulationsmechanismus der Cholesterin Homöostase. Der letzte Mechanismus, der durch die Oxysterole reguliert wird, besteht in der erhöhten Cholesterinveresterung zur Cholesterinzellspeicherung. Diese, sowie andere Oxysterol vermittelte Mechanismen, die nicht zur Cholesterinreguliergung beitragen, haben sich in den letzten Jahren als wichtige humanphysiologische Regulationsmechanismen etabliert. So wurde zum Beispiel die antivirale Eigenschaft von 25-hydroxycholesterol (25-OHC) entdeckt. Dies veranschaulicht, dass die physiologische Bedeutung der Oxysterole unterschätzt wurde und dass ihre Auswirkungen noch nicht vollständig verstanden werden. Dies hat auch damit zu tun, dass die Analytik der Oxysterole kompliziert ist und durch mehrere problematische Eigenschaften der Oxysterole behindert wird. Die Schwierigkeiten bei der Analytik sind unter anderem der große Cholesterinüberschuß, die geringen endogenen Oxysterolkonzentrationen, die mögliche Autoxidation von Cholesterin oder der Oxysterole selbst, die möglichen sonstigen chemischen Modifikationen, wie Veresterung, Reduktion oder Sulfatierung, die Lokalisierung in den Membranen, und schließlich ihre inerte chemische Struktur. Alle diese Faktoren, erschweren die Massenspektrometrische (MS) -Analyse und machen sie realtiv unspezifisch.

In dieser Arbeit wurden zwei Methoden für die Analyse von Oxysterolen in humanem Plasma mittels Flüssigchromatographie gekoppelt mit Tandem-Massenspektrometrie (LC-MS/MS) etabliert. Die erste Methode wurde für die Analyse von nicht-enzymatischen Oxysterolen im klinischen Kontext der Niemann-Pick Typ C (NP-C) Krankheitsdiagnose entwickelt. Die zweite Methode wurde für die Erforschung der Rolle von enzymatisch generierten Oxysterolen bei chronisch entzündlichen Darmerkrankungen (IBD) generiert.

In dem ersten Teil, der sich mit der Oxysterolanalyse für die NP-C Diagnose befasst, waren wir in der Lage, Cholestan-3β,5α,6β-triol (C-Triol) und 7-Ketocholesterol (7-KC) nach einer Steglich-Veresterung präzise, richtig und robust zu messen. Wir haben Referenzintervalle für die NP-C Diagnose erstellt, welche unabhängig von Alter oder Geschlecht sind und haben die prä-analytische Stabilität definiert. Wir fanden eine hohe intraindividiuelle Variation für beide Analyten, konnten jedoch erhöhte C-Triol Werte in allen Niemann-Pick - Patienten einschließlich Niemann-Pick Typ A und B - Patienten zeigen. Unsere Ergebnisse deuten darauf hin, dass vor allem C-triol ein Biomarker für alle drei Niemann-Pick Krankheiten ist.

Im zweiten Teil, der sich mit dem Oxysterol-Fingerabdruck in IBD befasst, waren wir in der Lage in einem chromatographischen Lauf 24(S)-hydroxycholesterol (24(S)-OHC), 25-hydroxycholesterol (25-OHC), 27-hydroxycholesterol (27-OHC), 7α,24(S)-dihydroxycholesterol (7α,24(S)-OHC), 7α,25-dihydroxycholesterin (7α, 25-OHC), 7α,27-Dihydroxycholesterin (7α,27-OHC), 7β,25-dihydroxycholesterin (7β,25-OHC) und 7β,27-dihydroxycholesterin (7β,27-OHC) aufzutrennen und zu quantifizieren. Unser bedeutenstes Ergebnis ist die Verminderung von 27-OHC und möglicherweise auch von 25-OHC im Plasma von IBD-Patienten im Vergleich zu gesunden Freiwilligen. Beide Monohydroxycholesterine sind Liganden der LXR Rezeptoren deren positiver Einfluss bei Entzündungen bereits in der Literatur beschrieben wurde. Die gemessene Verminderung der Liganden könnte somit die Entzündungsmechanismen bei IBD beeinflussen. Die Analyse der Mono- und

Dihydroxycholesterine als Biomarker für IBD ist ein neuer viel versprechender Ansatz, der allerdings mehr IBD Proben benötigt, um weitere Unterschiede zwischen den verschiedenen Studiengruppen d.h. der Kontrollgruppe, der aktiven und inaktiven Morbus Crohn und der aktiven und inaktiven Colitis Ulcerosa Gruppe aufzuzeigen.

INDEX

ABBREVIATIONS

24(S),25-EC:	24(S),25-epoxycholesterol
24(S)-OHC:	24(S)-hydroxycholesterol
25-OHC:	25-hydroxycholesterol
27-OHC:	27-hydroxycholesterol (also known as (25R)-26-hydroxycholesterol)
4β-OHC:	4β-hydroxycholesterol
7-KC:	7-ketocholesterol
7α,24(S)-OHC:	7α,24(S)-dihydroxycholesterol
7α,25-OHC:	7α,25-dihydroxycholesterol
7α,27-OHC:	7α,27-dihydroxycholesterol
7α-OHC:	7α-hydroxycholesterol
7β,25-OHC:	7β,25-dihydroxycholesterol
7β,27-OHC:	7β,27-dihydroxycholesterol
7β-OHC:	7β-hydroxycholesterol
ABCA1:	ATP-binding cassette proteins, subfamily A, member 1
ABCG1:	ATP-binding cassette protein, sub-family G, member 1
ABCG5:	ATP-binding cassette protein, sub-family G, member 5
ABCG8:	ATP-binding cassette protein, sub-family G, member 8
ACN:	Acetonitrile
APCI:	Atmospheric pressure chemical ionisation
Apo (A1 or E):	Apoliporotein A1 or E
BHT:	Butylated hydroxytoluene
CD:	Crohn disease
CE:	Collision energy
CH25H:	Cholesterol 25 hydroxylase
CI:	Confidence interval
COPII:	Coat protein complex II
Cps:	Counts per second
C-triol:	Cholestane-3β,5α,6β-triol

CTX:	Cerebrotendinous xanthomatosis
CV:	Coefficient of variation
CVw	Coefficient of variation for intra-individual variation
CXP:	Cell exit potential
CYP:	Cytochrome P450
CYP27A1:	Serol 27-hydroxylase
CYP46A1:	Cholesterol 24-hydroxylase
CYP7A1:	Cholesterol 7α-hydroxylase
CYP7B1:	Oxysterol 7α-hydroxylase
DP:	Declustering potential
EBI2 or GPR183:	Epstein-Barr virus-induced G-protein coupled receptor 2
EP:	Entrance potential
ER:	Endoplasmic reticulum
ESI:	Electrospray
EtOH:	Ethanol
FA:	Formic acid
GC:	Gas chromatography
GC-MS:	Gas chromatography coupled to mass spectrometry
HDL:	High density lipoprotein
HMGCR:	3-hydroxy-3-methyl-glutaryl- coenzyme A reductase (HMG-CoA reductase)
HPLC:	High performance liquid chromatography
HPLC–MS/MS:	High-performance liquid chromatography coupled to tandem mass spectrometry
HQC:	High quality control (endogenous level with 100 ng/mL standard)
IBD:	Inflammatory Bowel Diseases
IFN:	Interferons
INSIG:	Insulin-induced gene protein
IS:	Internal standards
IUPAC:	International union of pure and applied chemistry
LC-MS/MS:	Liquid chromatography coupled to tandem mass spectrometry
LDL:	Low density lipoprotein

LOD:	Limit of detection
LOQ:	Limit of quantification
LQC:	Low quality control (endogenous level)
LXR:	Liver X receptor
MeOH:	Methanol
MQC:	Medium quality control (endogenous level with 30 ng/mL standard)
MRM:	Multiple reaction monitoring
MS:	Mass spectrometry
NP-A:	Niemann-Pick type A
NP-B:	Niemann-Pick type B
NP-C:	Niemann-Pick type C
ORP:	OSBP-related proteins
POIS:	Performance optimising injection sequence
PUFA:	Polyunsaturated fatty acids
QC:	Quality controls
RORγt:	RAR (Retinoic acid receptor)-related orphan receptor gamma t
ROS:	Reactive oxygen species
S/N:	Signal to noise ratio
SCAP:	SREBP cleavage-activating protein
sER:	Smooth endoplasmic reticulum
SPE:	Solid-phase extraction
SREBP:	Sterol regulatory element binding protein
TOF-MS:	Time of flight mass spectrometry
UC:	Ulcerative colitis
UHPLC:	Ultra high performance liquid chromatography
UPC^2:	Ultra performance convergence chromatography
VLDL:	Very low density lipoprotein
XIC:	Extracted ion chromatogram

1. INTRODUCTION

1.1 BACKGROUND ON OXYSTEROLS

1.1.1 DEFINITION

Oxysterols are cholesterol oxidation derivatives with 27 carbon atoms, carrying an alcohol (ex: 7β-hydroxycholesterol (7β-OHC) **Figure 1A**), a carbonyl (ex: 7-ketocholesterol (7-KC) **Figure 1B**) or an epoxide group (ex: 5,6-epoxycholesterol **Figure 1C**) [1,2]. The oxysterol common name indicates at which carbon number the cholesterol backbone is oxidised. Oxysterols are either categorised according to their origin or, although overlapping with this first categorisation, according to the oxidation side either on the ring or on the side-chain.

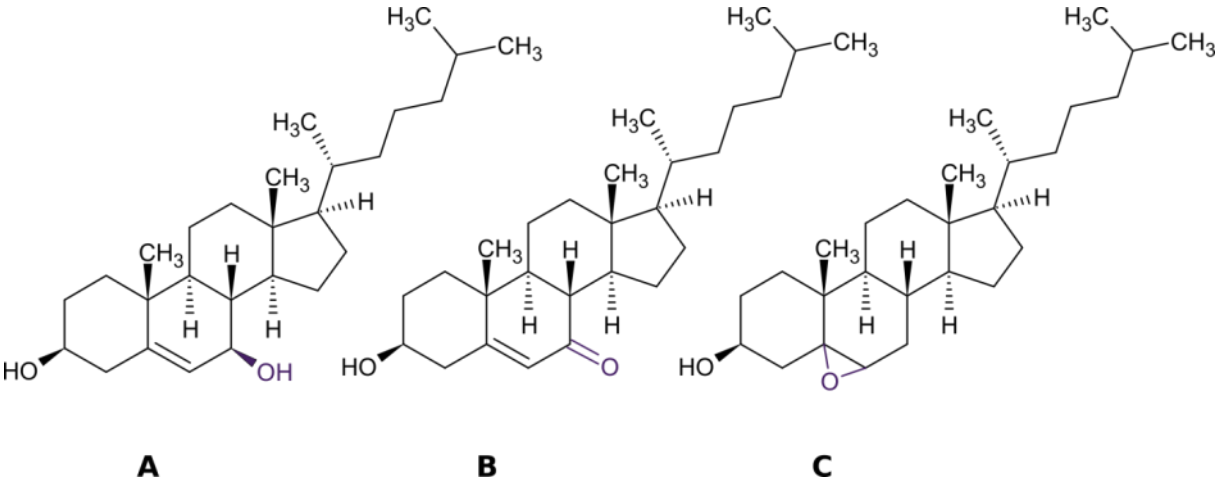

FIGURE 1| Three examples of different oxysterols A. 7β-hydroxycholesterol B. 7-ketocholesterol (7-KC), Figure C. 5,6-epoxycholesterol.

1.1.2 CLASSIFICATION AND CHEMICAL STRUCTURE

According to the LIPID MAPS classification, oxysterols are members of the sterol lipid family with the LIPID MAPS number ST0101 [3, 4]. The International union of pure and applied (IUPAC's) broad sterol definition is "Sterols are steroids carrying a hydroxyl group at C-3 and most of the skeleton of cholestane. Additional carbon atoms may be present in the side chain "[5]. The best known sterol example is cholesterol ((3β)-cholest-5-en-3-ol), from which the oxysterols directly derive **(**

Figure 2**)**.

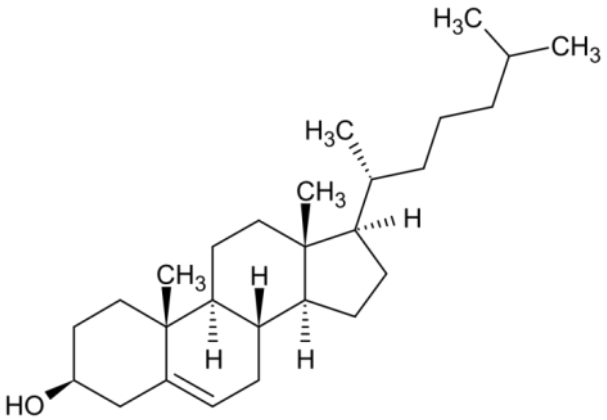

Figure 2| Illustration of the chemical structure of cholesterol

Sterols, together with bile acids and steroid hormones, belong on their turn to the steroid compound class. Steroids are defined by IUPAC as "compounds possessing the skeleton of cyclopenta[α]phenanthrene or a

skeleton derived therefrom by one or more bond scissions or ring expansions or contractions. Methyl groups are normally present at C-10 and C-13. An alkyl side chain may also be present at C-17" [5].

1.1.3 NOMENCLATURE

Oxysterols nomenclature follows the IUPAC convention for steroids [5] **(Figure 3)**.

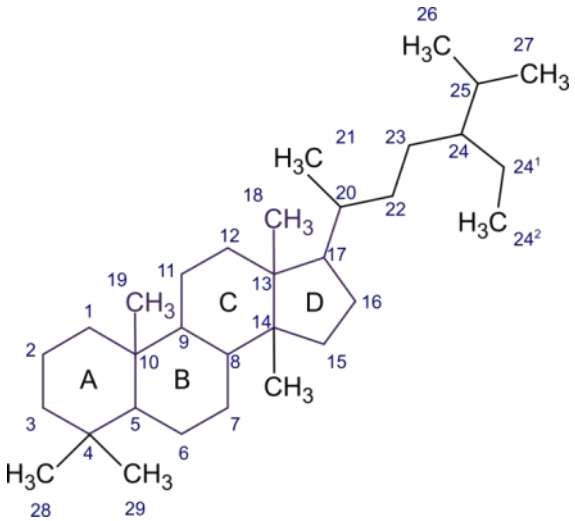

Figure 3| Sterol backbone with ring labelling and carbons numbering according to the IUPAC nomenclature. Ring denomination is labelled in black, carbon numbers are labelled in blue and the cyclopenta[α]phenanthrene is highlighted in dark purple.

It is to mention that the orientation of the hydrogen at position C-5 depends on the position of ring A towards ring B. If the A/B ring configuration is in *trans* then the C-5 hydroxyl is in *alpha*. In contrary, A/B *cis* configuration results in C-5 in *beta* position [6]. Generally, the C-5 hydrogen atom of sterols is in *alpha* position, while the C-5 hydrogen atom of bile acids is in *beta* position [6,7]. Steroid hormones do not have a hydroxyl group at C-5, but a double bond between C-4 and C-5. In the IUPAC guideline for nomenclature of steroids, the authors emphasis that "If one of the two methyl groups attached to C-25 is substituted it is assigned the lower number; if both are substituted, that carrying the substituent cited first in the alphabetical order is assigned the lower number" [5]. However, this last simple rule was violated by many scientists and manufacturers of chemicals, so that (25R)-26-hydroxycholesterol is now known and referred to as 27-hydroxycholesterol. R. Fakheri and N. Javitt counteracted by publishing in 2012 a review on the nomenclature and stereochemistry of 26-hydroxylated sterols explaining the misnaming of 27-hydroxycholesterol, nevertheless without noticeable acceptance from the readers [8]. In this manuscript we will use the name of 27-hydroxycholesterol since it is now the most commonly used denomination, especially in the medical literature and PubMed.

1.1.4 HISTORICAL PERSPECTIVE

At the beginning of the 20th century the chemist I. Lifschütz extracted from sheep wool wax a cholesterol- rich substance mixture which he called Eucerit. It was the first water-in-oil emulsifier and when added with paraffin it becomes a perfect basis for salve products called Eucerin. J. Lifschütz patented his discovery in 1902 and continued his research on Eucerin leading to the development of the worldwide famous Nivea Cream. During his experimentation he observed a novel substance class, which he named "Oxycholesterin", later known as oxysterols [9]. The oxysterol research era was born. Since then many discoveries were made and the main milestone discoveries are summarised in **Table 1.**

Table 1| Selected discoveries of oxysterols research milestones. The abbreviations and acronyms are explained in the chapter. Table adapted from Gill *et al*. (2008) [10].

Year	Discovery	Reference
1769	Cholesterol isolation by Poulletier de la Salle	11
1816	Cholesterol characterisation and naming by Chevreul	12
1906	Oxysterol discovery by Lifschütz	9,11
1927-28	Windaus and Wieland Nobel Prize awarding for elucidation of cholesterol and bile acids structures	13
1933	Cholesterol negative feedback regulation	14
1953	24(S)-OHC (cerebrosterol) discovered in human brain	15
1956	Oxysterols of enzymatic origin	16
1964	Bloch and Lynen Nobel Prize awarding for elucidating the cholesterol and fatty acid biosynthetic pathways.	13
1978	Oxysterol hypothesis on negative feedback regulation	17
1985	Brown and Goldstein Nobel Prize awarded for elucidating the regulation of cholesterol metabolism, including the LDL receptor discovery	13
1992	Discovery of the oxysterol binding proteins (OSBP)	18
1993	SREBP (master regulators of cholesterol homeostasis) activation inhibition by certain oxysterols	19
1994	27-OHC provides a cholesterol elimination mechanism for macrophages	20
1996	24(S)-OHC provides a cholesterol elimination mechanism for brain	21
2003	Accelerated HMGCR degradation by oxysterols and sterol intermediates of cholesterol synthesis	22
2007	Oxysterols are LXRs ligands & Insig identification as oxysterol sensor in the ER	23,24
2008	24(S),25-epoxycholesterol as acute regulator for smoothing cholesterol homeostasis response	25
2010	C-triol and 7-KC as biomarker for NP-C	26
2011	7α,25-OHC as EBI2 ligand for B cell homing within a lymph node	27,28
2013	Indications that oxysterols are implicated in IBDs	29

In 2010 to follow the rapid expansion of oxysterol research, several groups associated to form the European Network on Oxysterols Research (ENOR). It is a self-promoting and self-sustaining oxysterols organisation with about 80 members open to any research group worldwide and to any oxysterol research subdomain [2,30]

1.1.5 FUNCTION

Oxysterols are molecules present in the cell in very small amounts but exert multiple functions which are discussed in detail in the next subchapters. Long-time considered as cholesterol autoxidation side product they were of no concern but since more and more functions were discovered, they gained interest in the scientific community **(Figure 4)**. First, the main function of oxysterols in cholesterol homeostasis will be discussed [1,31–33]. Second, the recent oxysterol research is explained, which focuses on their implication in immunology with still many unanswered questions [28,34–36]. Finally, the focus will be on the implication of oxysterols in other physiological pathways, which indicates that oxysterols play a more important role in human physiology than initially expected.

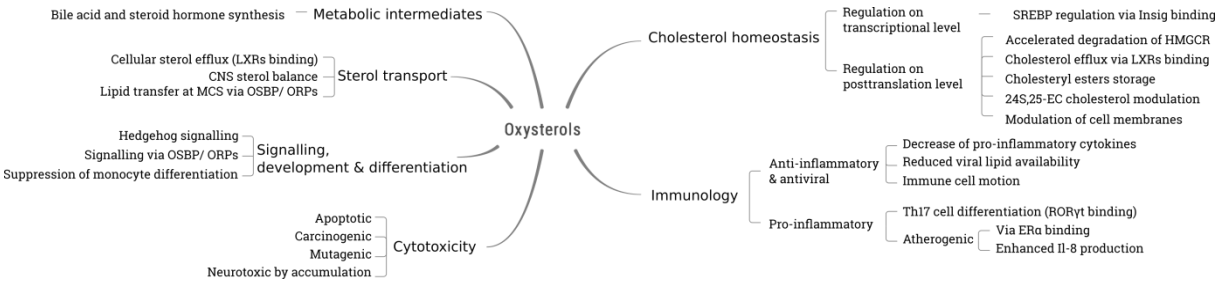

Figure 4| Overview of current functions attributed to oxysterols. Oxysterols regulate cholesterol homeostasis but they are also implicated in immunology, they have cytotoxic properties, are involved in signalling, development and cell differentiation, in sterol transport and finally they are metabolic intermediates. The abbreviations and acronyms are explained in the chapter. Adapted from Olkkonen *et al*. (2012) [37].

1.1.5.1 OXYSTEROLS REGULATING CHOLESTEROL HOMEOSTASIS

Cholesterol is a key component in living organisms and has an essential role in membrane structure, is a precursors for steroid hormones and bile acids, influences signalling processes and can be covalently attached to proteins [38,39]. Despite its crucial functions too much of it is deleterious and therefore its levels need to be tightly controlled. In this subchapter, cholesterol's own feedback regulation with the oxysterol contribution to it will be explained in detail. Cholesterol's own feedback regulation was discovered in the beginning of the 19th century by Schoenheimer and Breusch [14]. However, the oxysterol contribution to this process was only identified nearly half a decade later by Kandutsch *et al.* [17]. Nowadays oxysterols, in particular 25-hydroxycholesterol (25-OHC), 24(S)-hydroxycholesterol (24(S)-OHC) and 27-hydroxycholesterol (27-OHC), are known to be implicated in cholesterol homeostasis by the mean of different regulatory pathways acting on cellular cholesterol uptake, synthesis and efflux [1] **(Figure 5)**.

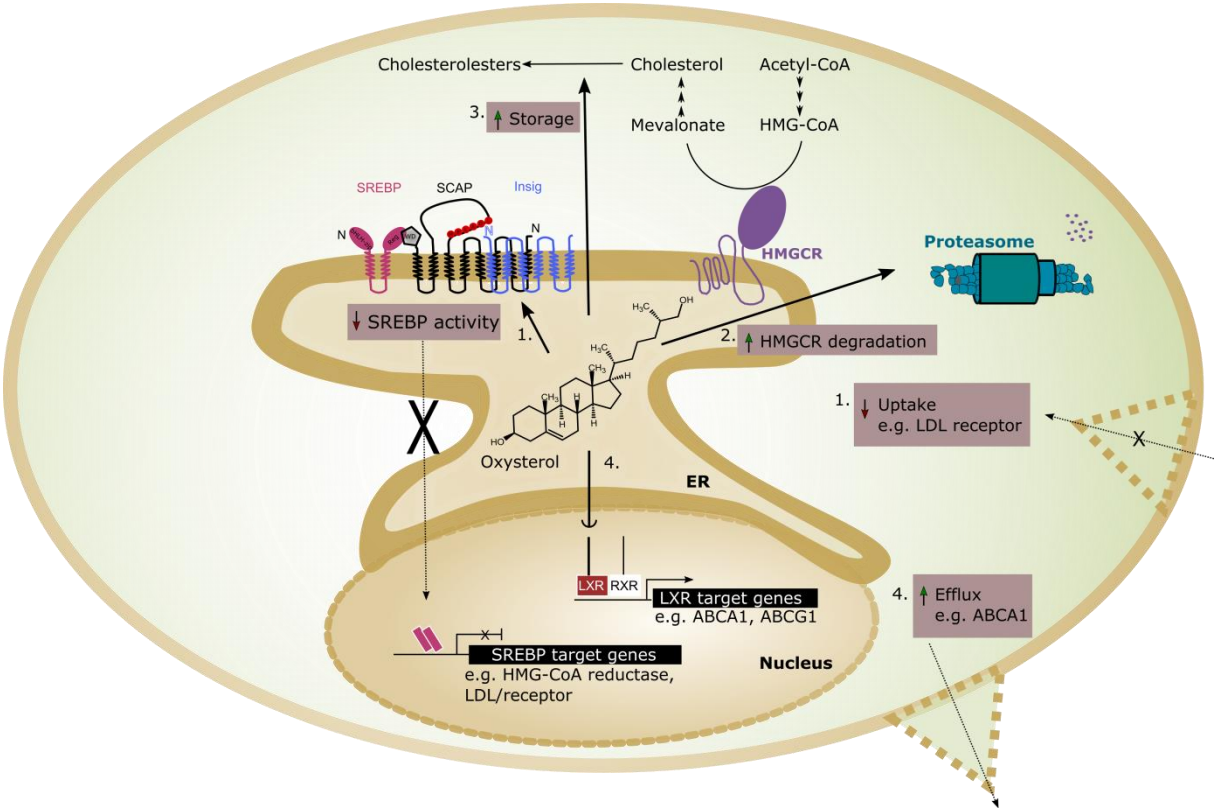

Figure 5| General overview on cholesterol homeostasis regulated by oxysterols. Oxysterols inhibit SREBP activity which reduces cellular cholesterol uptake (1.), they increase HMGCR degradation (2.) and cholesterol cell efflux (4.) and also increase the storage of cholesterol in form of cholesteryl esters (3.). The abbreviations and acronyms are explained in the chapter. Figure adapted from Brown *et al.* (2009) [1].

1.1.5.1.1 CHOLESTEROL HOMEOSTASIS REGULATION ON A TRANSCRIPTIONAL LEVEL BY OXYSTEROLS

The transcription factor sterol regulatory element binding protein (SREBP) acts on many genes directly involved in cholesterol, fatty acid biosynthesis, and low density lipoprotein (LDL) cell endocytosis [40]. These regulated genes exhibit the sterol responsive element (SRE) motif necessary for SREBP recognition and binding, leading to the activation of gene transcription [19,41].

The activity of SREBP is tightly regulated. At high sterol levels, cholesterol binds to SREBP cleavage-activating protein (SCAP) which retains SREBP in the membrane of the endoplasmic reticulum (ER) thereby suppressing SREBP gene activation [42] **(Figure 6A)**. This is the classical cholesterol feedback regulation mechanism. However, it

is also possible that in the same condition oxysterols, like 25-OHC, bind to Insulin-induced gene 1 protein (Insig) which is a third ER membrane protein coupled to the SCAP - SREBP complex thereby also holding back SREBP [23,43–45]. Whether cholesterol binding directly to SCAP or oxysterols binding to Insig, the outcome is exactly the same: SREBP is retained in the ER membrane and cannot act as transcription factor [46].

In the opposite situation, when cholesterol levels fall under a critical threshold, the SCAP-SREBP complex dissociates from Insig and moves via a coat protein complex II (COPII) vesicle transport to the Golgi [44,45,47,48] **(Figure 6B 1-2)**. Once located in the Golgi membrane SREBP will be sequentially cleaved by two proteases, site-1 protease (S1P) and site-2 protease (S2P), releasing the basic helix-loop-helix Zip type (bHLH-Zip) domain of SREBP, which translocate to the nucleus and activates target gene transcription [42,49] **(Figure 6B 3-4)**.

This description is a simplified version of a very complex process. In order to make the cholesterol homeostasis regulation more comprehensive, the different actors will be discussed in more details here below.

In the cell, cholesterol is to 95 % present in the plasma membrane or resides in lipid droplets. ER is the organelle with very little cholesterol, thus being the perfect cholesterol sensor in the cell [39]. Cholesterol concentration detection is mediated in the ER membrane by SCAP at its sterol-sensing domain in the N-terminus consisting of 5 of SCAP's 8 transmembrane regions [51–53]. An important hexapeptide sequence called MELDAL standing for methionine- glutamic acid- leucine- alanine- aspartic acid- leucine is located at SCAP's cytoplasmic loop 6 [54]. At the C-terminus a regulatory region with 4 motifs called WD40 physically interacts with SREBP [42]. During cholesterol deprivation, the MELDAL sequence is accessible for Sec24 binding, a component of the Sar1/Sec23/Sec24 complex of COPII coat proteins [54,55]. Through this specific binding the SCAP-SREBP complex is sequestered in COPII- coated vesicles that leave the ER for the Golgi. When the sterol level is rising and cholesterol is binding to SCAP or oxysterols to Insig, loop 6 of SCAP changes its conformation. The consequence is that the MELDAL sequence binds to Insig and can no longer be recognised by the COPII proteins thus abrogating the transport pathway [55].

Insig is considered to be a small ER retention protein, consisting of 6 transmembrane domains. Insig-oxysterol interaction takes place at the third and fourth transmembrane span, while SCAP binding occurs at the cytosolic loop between transmembrane domains 4 and 5. Insig has two isoforms: Insig-1 and Insig-2 [44,45]. Insig-1 is a 277 amino acid long protein highly expressed in human liver, while Insig-2 contains 255 amino acids and is ubiquitously expressed. Insig-1 expression is transcriptionally controlled by SREBP meaning that cholesterol biosynthesis is accompanied by the synthesis of one of its own regulatory elements [40,56]. In contrast, the Insig-2 promoter is activated by insulin receptor signalling [43–45]. Oxysterol binding to the Insig proteins has a second effect besides ER retention of the SCAP-SREBP complex [23]. Upon oxysterol binding, Insigs are able to interact with 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase or HMGCR), the rate-limiting enzyme in cholesterol biosynthesis also located in the ER membrane. Insig's binding to HMGCR causes its degradation by the ubiquitin-proteasomal system [22,57–59].

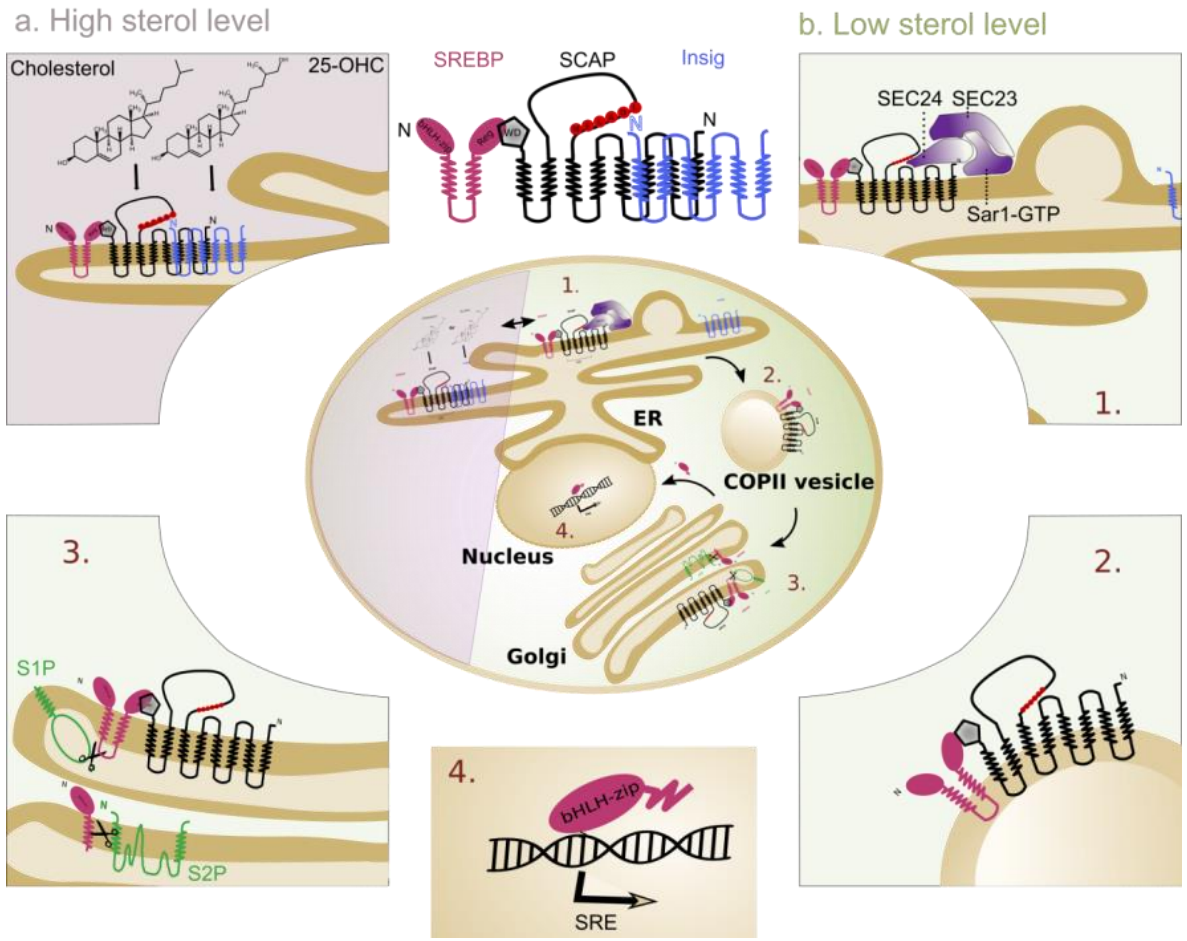

Figure 6| Cholesterol feedback mechanism. At high sterol level, the Insig-SCAP-SREBP complex is retained in the ER (A.). At low sterol levels, Insig dissociates from the SCAP-SREBP complex and the MELDAL sequence (highlighted in red on SCAP) is accessible for Sec24 binding, thus being a starting point for vesicle formation (B.1). SCAP-SREBP are via COPII vesicle transported from the ER to the Golgi (B.2). In the Golgi the SCAP-SREBP complex is sequentially spliced by S1P and S2P to deliver the SREBP transcription factor (B.3). The SREBP transcription factor moves into the nucleus activating genes at the SRE recognition site (B.4). The abbreviations and acronyms are explained in the chapter. Figure adapted from Ikonen *et al.* (2008) [50]

SREBP is a 2 transmembrane protein with its C-Terminus interacting with SCAP. The N-Terminus consists of a transcription factor motif of the basic helix-loop-helix Zip type [60]. Both terminuses are exposed to the cytosolic side. SREBP is encoded by two distinct genes generating SREBP-1 and SREBP-2 proteins [61,62]. SREBP-1 expression can be activated by high cellular sterol content binding to liver X receptors (LXRs), while SREBP-2 is not [62,63]. In addition SREBP-1 exists under two isoforms: SREBP-1a and 1c, both controlling genes of fatty acids synthesis and each a specific set of genes [63,64]. SREBP-1a regulates also genes of the cholesterol biosynthetic pathways whereas SREBP-1c regulates genes involved in glucose utilisation [40,65]. SREBP-2, is the predominant transcription factor in liver, regulating genes involved in cholesterol homeostasis including genes encoding cholesterol biosynthetic enzymes and genes responsible for LDL receptor expression [40]. SREBPs are finally activated after translocation to the Golgi by the action of two Golgi-associated membrane bound proteases [49,66]. SCAP stimulates the first cleavage mediated by site-1 protease, a serine protease, at the luminal loop between the two transmembrane domains [67]. The second cleavage is mediated by the site-2 protease, a Zn^{2+} metalloprotease [68] and results in the cytosolic release of the N-terminal the basic helix-loop-helix Zip type motif. This mature transcription factor migrates into the nucleus and activates its target genes [49,68].

1.1.5.1.2 CHOLESTEROL HOMEOSTASIS REGULATION ON A POSTTRANSLATIONAL LEVEL BY OXYSTEROLS

1.1.5.1.2.1 ACCELERATED DEGRADATION OF THE HMG-COA REDUCTASE

As already highlighted during the description of Insig functions, oxysterol-activated Insig interaction leads to HMGCR destruction by the ubiquitin-proteasomal system **(Figure 7).** Insig associates with ubiquitin ligases gp78 (= autocrine motility factor receptor (AMFR)) and translocation in renal cancer from chromosome 8 (Trc8), both responsible for HMGCR ubiquitination. This ubiquitination is then recognised by ATPases associated with various cellular activities - Valosin-containing protein (AAA-ATPase VCP/p97) extracting the HMGCR across the ER membrane. Ubiquinated HMGCR released into the cytosol will then be degraded in the proteolytic chamber of the 20S proteasome [69]. This captivation, extraction and destruction process is called ER-associated degradation (ERAD) [70,71]. It was shown that the ubiquitin-proteasomal degradation pathway is also activated on a posttranslational level. Mevalonate-derived metabolites like geranylgeraniol are known to destabilise HMGCR [58,72,73] and it was recently shown that they increase the sterol-accelerated ERAD of HMGCR by a still badly understood mechanism but do not affect sterol-induced ubiquitination [74]. The transcriptional and posttranslational regulations of HMGCR demonstrate the need for a tight control of this rate-controlling enzyme in cholesterol biosynthesis.

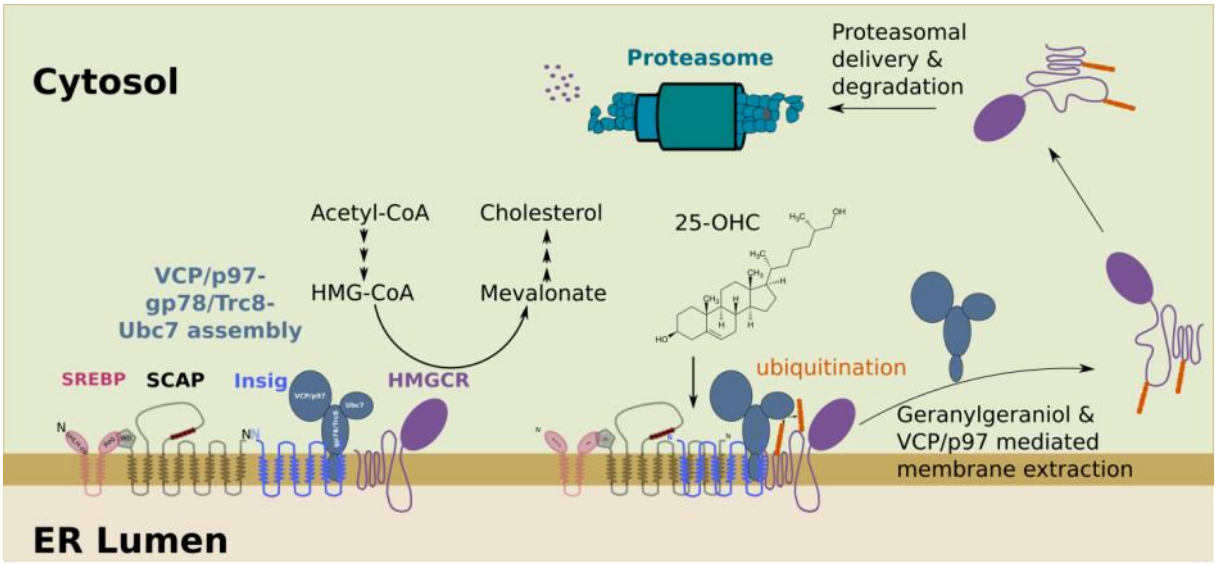

Figure 7| Posttranslational HMGCR degradation mechanism. Oxysterols induce HMGCR ubiquitination being a molecular signalling for HMGCR membrane extraction and subsequent degradation by the proteasome. The abbreviations and acronyms are explained in the chapter. Figure adapted from Schumacher *et al.* (2015) [74].

1.1.5.1.2.2 INCREASED CHOLESTEROL EFFLUX BY LIVER X RECEPTORS ACTIVATION

LXRs were declassified as orphan nuclear receptors when it was shown that oxysterols are their natural ligands [24,75]. LXRα and LXRβ dimerise with the obligate partner 9-*cis* retinoic acid receptor α (RXRα) followed by LXR response element binding in the promoter region of LXR target genes [76,77]. The heterodimer senses cellular oxysterols and their binding provokes conformational changes of LXR, releasing in that way co-repressors and recruiting co-activators for gene activation [78–81].

Up-regulated genes are those that control sterol and fatty acid metabolism including genes involved in reverse cholesterol transport [82]. The main actors of reverse cholesterol transport activated by LXRs are: ATP-binding cassette protein, sub-family A, member 1 (ABCA1), ATP-binding cassette protein, sub-family G, member 1 (ABCG1), ATP-binding cassette protein, sub-family G, member 5 (ABCG5) and ATP-binding cassette protein, sub-family G, member 8 (ABCG8), as well as apolipoprotein E (apoE), cholesteryl ester transfer protein (CETP), phospholipid transfer protein (PLTP), and scavenger receptor B1 (SR-B1) [82].

In reverse cholesterol transport, the ATP-binding cassette transporters such as ABCA1 and G1 of macrophages facilitate the charging of cell phospholipids and cholesterol onto exchangeable apolipoproteins, especially on apoA-I, to initiate and propagate high density lipoprotein (HDL) formation [83,84]. The cholesterol of the growing HDL is subsequently converted into cholesteryl esters by the lecithin-cholesterol acyltransferase (LCAT) and further transported by the mean of three possible mechanisms. The first and most important mechanism in humans is the selective uptake of HDL-derived cholesteryl esters by the liver through the scavenger receptor B1 [85]. The second mechanism is the exchange of cholesteryl esters from HDL to VLDL and LDL by the cholesteryl ester transfer protein. Consequently, the second mechanism allows the third mechanism of liver uptake of (V)LDL- derived cholesteryl esters through the LDL receptors [63]. The liver and the intestine expresses ABCG5 and ABCG8, which are half-transporters that form obligate heterodimers allowing the cholesterol export into the bile, respectively in the intestinal lumen, thus leading to the cholesterol excretion from the body [86–88]. Cholesterol conversion into bile acids is in rodents additionally enhanced through the LXRs, since LXRs control and activate the rodent cholesterol 7α-hydroxylase (CYP7A1), being the rate-limiting enzyme of the ‘‘classical’’ bile acid synthesis pathway [89].

An alternative to cholesterol body export is to store cholesterol as cholesteryl esters within lipid droplets, which is the cell’s dominant strategy in situation of excess cellular cholesterol [90]. The esterification occurs in the ER by the action of acetyl-coenzymeA -acetyltransferase (ACAT) [90]. Acetyl-coenzymeA -acetyltransferase possesses a catalytic and an allosteric activator binding site, with only cholesterol being able to strongly bind to the allosteric site. The molecular mechanism of oxysterol - activated cholesterol esterification is therefore still unclear [91].

1.1.5.1.2.3 24(S),25-EPOXYCHOLESTEROL AS MONITOR AND MODULATOR OF CHOLESTEROL SYNTHESIS

24(S),25-epoxycholesterol (24(S),25-EC) was identified to be an oxysterol with a particular role in the regulation of cholesterol homeostasis. Its origin is atypical since it is synthesised in a shunt of the mevalonate pathway, parallel to cholesterol synthesis. In the shunt pathway squalene epoxidase adds not one but two epoxide groups to squalene forming diepoxysqualene (DOS) [92,93]. 2,3-oxidosqualene cyclase (OSC) cyclises further both diepoxysqualene and monooxidosqualene (MOS) but with a preference for diepoxysqualene to give rise to the endproducts of 24(S),25-EC and cholesterol by the mean of multiple steps [94]. 24(S),25-EC is able to inhibit cholesterol synthesis similar to 25-OHC, 24(S)-OHC and 27-OHC by stimulating HMGCCR degradation, suppressing SREBP activation and stimulating cholesterol efflux. 24(S),25-EC in addition is able to inhibit the

desmosterol reductase (DHCR24), inhibiting so the desmosterol-cholesterol conversion [95]. Taking together all actions of endogenous 24(S), 25-EC indicates that this molecule is fine-tuning the acute control of cellular cholesterol [25] **(Figure 8)**.

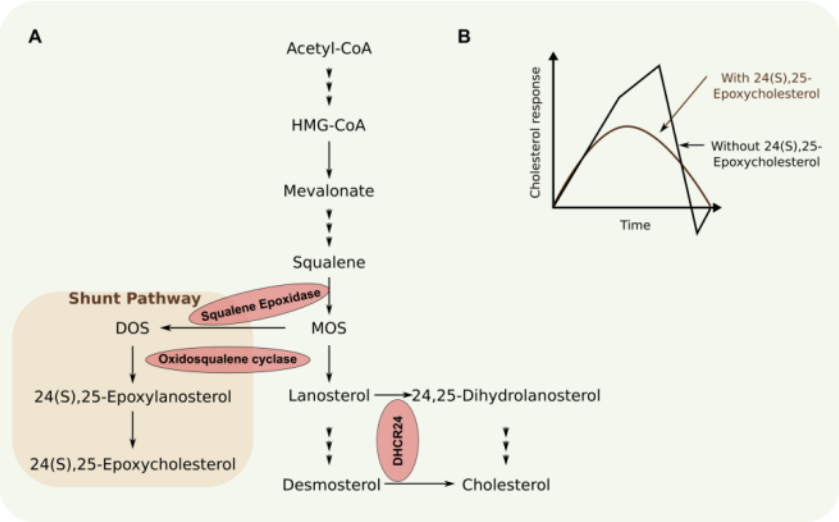

Figure 8| 24(S),25-EC's Shunt pathway (A). 24(S),25-EC is synthesised in parallel to cholesterol by squalene epoxidase by a second epoxide addition to squalene and further metabolisation by oxidosqualene cyclase (A). The hypothetical cholesterol response over time in absence (black line) and presence of 24(S),25-EC demonstrating the smoothing effect of the latest (B). The abbreviations and acronyms are explained in the chapter. Figure adapted from Gill *et al.* (2008) [10].

1.1.5.1.2.4 OXYSTEROL-MEMBRANE INTERACTIONS IN THE REGULATION OF CELLULAR CHOLESTEROL HOMEOSTASIS

Oxysterols are among others imbedded in plasma membrane but whether they exert any impact on the membrane structure was till recently unknown. Gale *et al*. were one of the first to show in 2009 that the "side chain oxysterols, but not steroid ring modified oxysterols, exhibit membrane expansion behaviour in phospholipid monolayers and bilayers *in vitro*" [96]. The presence of oxysterols in membrane reduces membrane-cholesterol affinity, although the mechanism behind it is not known [97–100]. Recently, Ory's group further elucidate the so called membrane expansion behaviour in showing that increased cholesterol liberation was due to 25-OHC presence in membrane resulting in membrane thinning [101].

1.1.5.1.3 BRAIN CHOLESTEROL HOMEOSTASIS

Cholesterol homeostasis regulation by oxysterols such as described in the previous sections cannot be applied to brain cholesterol homeostasis. The reason, is that cholesterol is unable to cross the blood-brain barrier thus limiting the exchange of cholesterol between the cerebrum and the periphery. However, central nervous cells, such as neurons and glial cells are extremely high cholesterol requestors meaning that the brain essentially depends on *de novo* synthesis and recycling [102]. In addition, the regulation of brain cholesterol homeostasis requires oxysterols since they are able to cross the blood-brain barrier in contrary to cholesterol. In brain, cholesterol can be metabolised into 24(S)-OHC, which is then able to leave the brain, accounting as the main brain efflux mechanism [21,103,104]. In the opposite direction, 27-OHC being the major plasma oxysterol, is able to enter the brain [105]. These two oxysterols are as already mentioned, LXRs agonists activating thereby the expression of cholesterol efflux transmembrane transporters [106]. LXRβ is highly expressed in the brain and through its activation; oxysterols regulate the cerebral cholesterol pool according the brain's needs [107–109]. Further regulatory roles of oxysterols in brain metabolism other than cholesterol homeostasis have been observed. A first example is the discovery of LXRα and β activation by oxysterols promoting myelination and remyelination in the cerebellum, while in the central nervous system (CNS) and peripheral nervous system

(PNS) the opposite effect was observed [110,111]. A second example, is the negative influence of 27-OHC on learning, for which the mechanism has not been solved [112].

1.1.5.2 OXYSTEROL PARTICIPATION IN IMMUNITY

"25-hydroxycholesterol may be more important as a regulator of immunity than as a regulator of cholesterol metabolism ", was Diczfalusy's provoking statement in his review on 25-OHC [113]. It is too early to judge whether this affirmation is true or not, but it highlights the different functions discovered in innate and adaptive immunity for 25-OHC and its metabolite 7α,25-dihydroxycholesterol (7α,25-OHC).

1.1.5.2.1 OXYSTEROL'S IMPLICATION IN THE ADAPTIVE IMMUNE SYSTEM

1.1.5.2.1.1 ANTI-INFLAMMATORY AND ANTIVIRAL PROPERTIES

25-OHC function in adaptive immunity is still controversial, however, it mainly appears to be anti-inflammatory and anti-viral **(Figure 9)**. The anti-inflammatory property of 25-OHC is currently thought to play a role during Type 1 Interferon's (IFNs) (IFN α and β) suppressive effects of the immune system [114]. This cytokine class is known to be a major host defence mechanism after Toll-like receptor (TLRs) activation by bacterial or viral ligands. Type 1 IFNs help to prevent uncontrolled inflammation by down regulating inflammasome's action and by reducing the production of pro-inflammatory IL-1 cytokines (IL-1α, IL-1β and IL-18) [115–117]. It was discovered that type 1 IFNs increase cholesterol 25 hydroxylase (CH25H) expression in the ER, consequently producing 25-OHC that suppresses SREBP activation [114,117,118]. In turn, reduced SREBP activity decreases the expression of IL-1β and the inflammasome activity, mediating the negative feedback pathway to prevent new IL-1 cytokine production and release [114]. How SREBP promotes IL-1β expression is yet not defined, and may be both independent and downstream of sterol biosynthesis. Currently, the effect is mainly believed to be of indirect nature, since no direct interaction between SREBPs and the IL-1β promoter could be found [34].

IFN dependent 25-OHC action of blocking of SREBP activation appears also to be the underlying mechanisms for the antiviral properties of 25-OHC. During infections, viruses alter host cellular lipid metabolism to support their own replication [119,120]. However, most viral infections are combated with a strong induction of IFNs increasing 25-OHC levels, which down regulate sterol biosynthesis and thus decrease virus entry into the cell and replication [121]. The exact pathway of 25-OHC antiviral effect is still under investigation and several hypotheses have been raised. Among them are altered cholesterol content of the plasma membrane, which could neutralise viral fusion and entry, or altered cholesterol content of internal membranes to avoid the reprogramming of organelles for viral assembly and packaging, or suppression of endogenous and viral protein prenylation which is involved in viral replication and assembly, and finally a possible direct inhibition of viral replication [121–124]. Even if more studies are required to understand 25-OHC antiviral mechanism, 25-OHC was proposed as a therapeutic option against viruses [122,125,126].

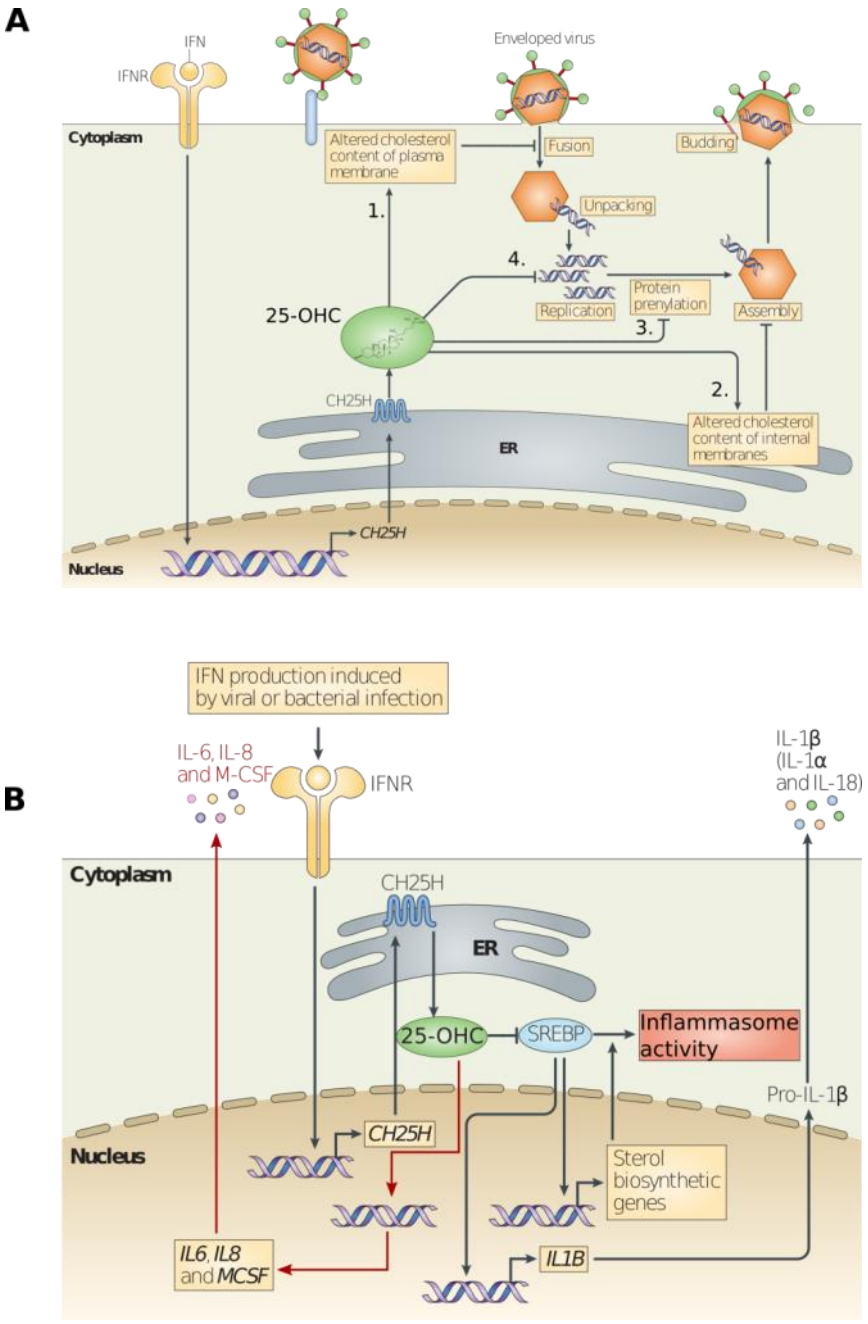

Figure 9| Oxysterols implication in immunity. Oxysterols were shown to have anti-viral properties (A) and anti- but also pro-inflammatory properties (B). The anti-inflammatory pathway is highlighted in black, while the pro-inflammatory pathway is highlighted in red (B). The abbreviations and acronyms are explained in the chapter. Figure adapted from Cyster *et al.* (2014) [34].

25-OHC implication in immunology is known since the 1970s but 7α,25-hydroxycholesterol (7α,25-OHC) participation was first published in two parallel studies in Nature in 2011 [28,35]. They described 7α,25-OHC as a ligand for the Epstein–Barr virus-induced gene 2 receptor (EBI2, also known as GPR183). EBI2 is a G-protein-coupled receptor connected with $G\alpha_i$ subunits, which triggers several signal transduction cascades upon ligand activation. Among the mobilised pathways are the activation of RHO family GTPases and mitogen-activated protein kinase (MAPK), including extracellular signal-regulated kinase (Erk) and p38- mitogen-activated protein kinase, and intracellular calcium flux [35,127–129]. EBI2 is expressed on B cells in the secondary lymph organs and *in vitro* and *in vivo* studies shed light on the involvement of the EBI2 receptor-7α,25-OHC system in the humoral immune response.

To summarise, B cells express the EBI2 receptor on their surface in the follicles of the secondary lymph organs. After antigen contact at the follicle T cell zone interface, B cells undergo a series of migratory steps that are

mediated by the concentration gradient of 7α,25-OHC. B cell motion towards distinct microenvironments in the follicle is crucial for induction, maturation and survival of plasmablasts and thereby for the activation of the immune response [34,35,130–133].

Given all these information of oxysterols role in adaptive immunity Cyster *et al.* hypothesised that 25-OHC and 7α,25-OHC operate in a hand-in-hand manner: Exposure to TLR ligands during viral infection increases 25-OHC making it more available for oxysterol 7α hydroxylase (CYP7B1) to convert it into 7α,25-OHC. 7α,25-OHC, as EBI2 ligand, will induce B cell motion in secondary lymph organs and in this way activate an immune response. Probably, the remaining 25-OHC quenches inflammasome activity and exerts its antiviral effects to limit cell damage [34].

1.1.5.2.1.2 OXYSTEROLS PRO-INFLAMMATORY ACTIONS

In contrary to the anti-inflammatory and antiviral properties of the oxysterols studied so far, also pro-inflammatory properties have been reported in the last years. At first, 25-OHC was shown to amplify the secretion of IL-6, IL-8 and macrophage colony-stimulating factor (M-CSF) in macrophages and in airway epithelial cells after infection by a double-stranded (ds) RNA virus [36,134]. At second, a very recent revelation was that retinoic acid receptor (RAR)-related orphan receptor gamma t (RORγt) binds to 7α,27-OHC and 7β,27-OHC [135]. The ligand-receptor binding showed to drive the differentiation of T_H17 cells producing IL-17 and thus initiating an immune response [135].

1.1.5.2.2 25-OHC IMPLICATION IN THE INNATE IMMUNE SYSTEM

Oxysterols are besides their role in adaptive immunity, also implicated in the innate immune system. Ecker *et al.* showed that 25-OHC suppressed the differentiation of monocyte into macrophages. Since macrophages produce and secrete 25-OHC in response to TLR activation, it may be a negative-feedback mechanism that reduces the number of phagocytes capable of synthesising 25-OHC [136,137].

1.1.5.3 OXYSTEROLS IMPLICATION IN DIFFERENT PHYSIOLOGICAL MECHANISMS

1.1.5.3.1 OXYSTEROL BINDING PROTEINS - RELATED PROTEINS (OSBP)

A conserved family of lipid transfer proteins, the OSBP-related proteins (ORP) discovered by the finding of oxysterol binding proteins require the binding of oxysterols for their functioning. However, ORPs do not only bind to oxysterols but to a variety of lipids such as phosphosinositides and phosphatidic acid. Their function consists in the transfer of sterols in exchange of phosphatidylinositol 4- phosphate at ER membrane contact sites (MCS) with other organelles. This intracellular sterol transport mechanism is thought to regulate lipid fluxes, organelle lipid compositions and cell signalling [138].

1.1.5.3.2 HEDGEHOG PATHWAY

The hedgehog pathway is important for embryonic pattern formation and adult tissue homeostasis [139]. Several studies found a link between oxysterols and the hedgehog pathway but they differ with respect to whether oxysterols are a hedgehog pathway activator or inhibitor [140–144]. Research on oxysterol's implication in the hedgehog pathway was based on three different theoretical links between the hedgehog pathway and sterols in general. The first argument is the covalent modification of the hedgehog protein by cholesterol to facilitate the protein anchoring to the plasma membrane. Here, the cholesterol availability for covalent protein modifications is probably regulated by the cholesterol homeostasis function of oxysterols [10,145]. Secondly, a sterol sensing domain (SSD), also found in the structure of proteins implicated in cholesterol homeostasis such as HMGCR, SREBP and SCAP, was detected in a hedgehog receptor named Patched [146,147]. Finally, disorders of

cholesterol biosynthesis like Smith-Lemli-Opitz Syndrome lacking the enzyme of the final cholesterol conversion step and resulting in the accumulation of 7-dehydrocholesterol, are associated with gross developmental abnormalities [141,142].

1.1.6 OXYSTEROL ANABOLISM AND CATABOLISM IN THE ORGANISM

1.1.6.1 OXYSTEROL ORIGINS

Oxysterols have three different possible origins: one exogenous through dietary intake and two others of endogenous origin, either due to cholesterol autoxidation or due to enzymatic transformations **(Figure 10)**. Although this holds for being generally true, exceptionally some oxysterols like 7α-hydroxycholesterol (7α-OHC), 4β-hydroxycholesterol (4β-OHC) and 25-OHC stem from both origins, meaning that they can be synthesised either enzymatically or non-enzymatically [148–150]. The cholesterol autoxidation products, which were discovered first, are mainly oxidised on their B-ring. Oxysterols metabolised by the enzymatic pathways are hydroxylated either at the side-chain or on their A or B-ring, depending on the enzyme and the tissue [151,152].

1.1.6.1.1 OXYSTEROLS FROM EXOGENOUS DIETARY SOURCE

Modern human's exogenous source of oxysterols derives especially from dairy, egg and meat products in the western cholesterol-rich diet. The oxysterols are thought to be generated by auto oxidation during food manufacturing like cooking, processing and storage [153]. The estimated oxysterol content in western diet varies from ppm ranges [154,155] to 1% [156]. The most representative food oxysterols are 7-KC, 7α-OHC, 7β-OHC, 5α,6α-epoxycholesterol, and 5β,6β-epoxycholesterol (5β,6β-EC) [153,157]. They are absorbed by the gut and carried on chylomicrons in the blood circulation [158–162]. Several absorption studies were made but the results varied from very low oxysterol absorption of 6 % [163] to extremely high oxysterol absorption of 93 % [161]. The discrepancy of the oxysterol absorption results might be explained by the use of different doses, models and vehicles used to administer the oxysterols [153]. Other hypotheses for the large difference are the preferential absorption of some oxysterols or eventually, the oxysterol hydrolysation directly in the gut [153]. The impact of dietary oxysterols on human health is unresolved. However, there is a tendency to consider them harmful, like Biasi *et al.*, who suggested oxysterols to be a potential trigger and/or worsening factor for Inflammatory Bowel Diseases (IBD) [29,164–166].

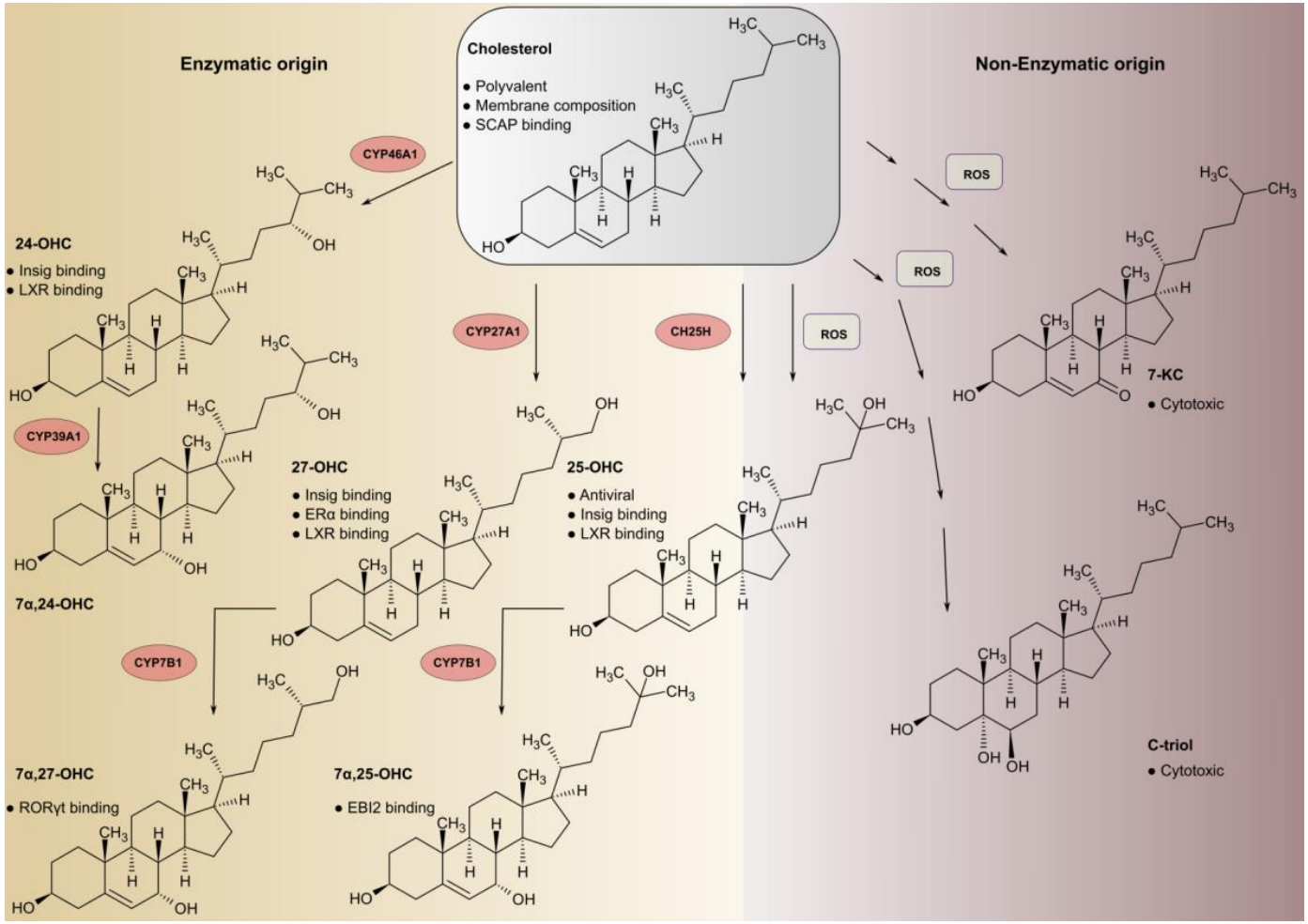

Figure 10| Oxysterols from enzymatic and non-enzymatic origins. Cholesterol is transformed to monohydroxycholesterols as for example 24(S)-hydroxycholesterol (24(S)-OHC), 27-hydroxycholesterol (27-OHC) and 25-hydroxycholesterol (25-OHC) through the enzymatic action of cholesterol 24-hydroxylase (CYP46A1), sterol 27-hydroxylase (CYP27A1) or cholesterol 25-hydroxylase (CH25H), respectively. 24-OHC is then converted to 7α,24(S)-dihydroxycholesterol (7α,24-OHC) by the action of the oxysterol 7α-hydroxylase 2 (CYP39A1), while 27-OHC and 25-OHC are both converted to their respective dihydroxycholesterols by the oxysterol 7α-hydroxylase (CYP7B1). Additionnaly to the enzymatic conversion, 25-OHC derives also from non-enzymatic origin through radical oxygen species (ROS). Other examples of non-enzymatic generated oxysterols are 7-ketocholesterol (7-KC) and cholestane-3β,5α,6β-triol (C-triol). The abbreviations and acronyms are explained in the chapter. Figure adapted from Cyster *et al*.(2014) [34].

1.1.6.1.2 NON-ENZYMATIC CHOLESTEROL OXIDATION

The non-enzymatic cholesterol oxidation mechanism is divided into two main pathways, although *in vivo* some exchanges between both pathways occur [167].

1.1.6.1.2.1 THE FREE RADICAL-MEDIATED CHOLESTEROL OXIDATION PATHWAY

The major non-enzymatic cholesterol oxidation pathway is mediated by radical oxygen species involving an initiation, propagation and an ending phase [1,166,167] **(Figure 11).**

The initiation phase consists of hydrogen abstraction by a free radical on the carbon with a labile hydrogen followed by oxygen capture [1,167]. In the case of cholesterol, the abstraction takes place at the allylic hydrogen atom at C-7. The generated C-7 centered radical is relatively long-lived and further reacts with molecular oxygen to form a cholesterol peroxyl radical [1]. Free radical-mediated cholesterol oxidation is triggered by radical oxygen species such as hydroxyl radical (HO●), peroxynitrite (ONOO-) or transition metals in a redox-active state [166].

The resulting cholesterol peroxyl radical is highly reactive and recruits further non-oxidised lipids, resulting in the formation of cholesterol hydroperoxides or other lipid peroxides restarting thus the chain reaction, also called propagation [167,168].

In plasma, in contrary to *in vitro* conditions, polyunsaturated fatty acids (PUFA) are more readily attacked by reactive oxygen species (ROS) than cholesterol [167]. This is the reason why polyunsaturated fatty acid autoxidation has been intensively studied in comparison to cholesterol oxidation but both are triggered by a similar mechanism. Oxidation on polyunsaturated fatty acids results in isoprostanes, regio-and stereoisomers of prostaglandins, while cholesterol oxidation gives rise to oxysterols [167]. Oxidation of both substances is usually described as separate events but occurs cooperatively at the cellular levels. The oxidation site for both entities is either in cell membranes or on the lipoproteins. It causes alterations in fluidity, permeability and structure. The propagation of the radical oxidation is facilitated by the more hydrophilic nature of the oxidation products than its parent molecules, which partition thereby more easily in more aqueous cell compartments on the intra-and intercellular level [167]. In addition, the dispersion step of the radical reaction was found to be facilitated by the sterol carrier protein-2 (SCP-2) which is responsible for intracellular lipid trafficking including lipid hydroperoxides, thus participating in the peroxidative damage [169].

The ending phase is engaged by either endogenous chain-breaking antioxidants such as α-tocopherol or by the enzyme phospholipid glutathione peroxidase (PHGPx), which converts cholesterol hydroperoxides into more stable hydroxyl-products [167,170]. A typical example of an end product of the free radical mediated cholesterol oxidation pathway is 7-KC [167,168] .

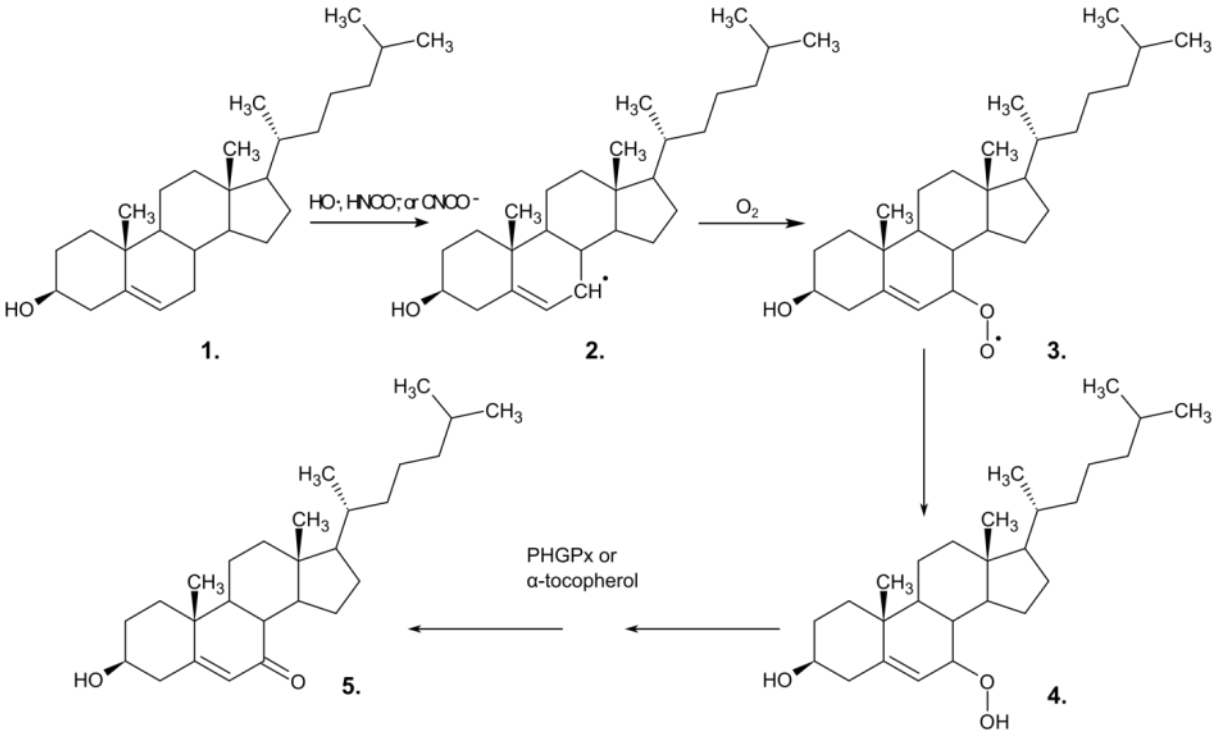

Figure 11| The free- radical mediated cholesterol oxidation pathway. Cholesterol (1.) is transformed by a free radical to a cholesterol (C7) carbon centred radical (2.). This highly reactive compound then reacts with molecular oxygen to result in a cholesterol peroxyl radical (3.), which further reacts to form a cholesterol hydroperoxide (4.). Finally, through the intervention of phospholipid glutathione peroxidase (PHGPx) and α-tocopherol the reactions is stopped and stable oxysterols as for example 7-KC are formed (5.). The abbreviations and acronyms are explained in the chapter. Figure adapted from Iuliano *et al.* (2011) [167].

1.1.6.1.2.2 NON-RADICAL MEDIATED CHOLESTEROL OXIDATION PATHWAYS

To date three non-radical mediated cholesterol oxidation mechanisms were described **(Figure 12)**. Singlet oxygen ($^1O_2$) is an important non-radical molecule involved in cholesterol oxidation and is formed when molecular oxygen receives an input of energy, for example by photoactivation [167]. Singlet oxygen's half-life is extremely short and when reacting with cholesterol gives rise to four primary oxysterols: the preferentially formed 5α-cholesterol-hydroperoxide, 6α-cholesterol-hydroperoxide, 6β-cholesterol-hydroperoxide and chol-dioxetane [166,167,171]. Allylic rearrangement may further occur on these primary oxysterols as for example the rearrangement of 5α-cholesterol-hydroperoxide to 7α-cholesterol-hydroperoxide or 7β-cholesterol-hydroperoxide [172,173].

Ozone (O_3) in lungs reacts with cholesterol to yield cholesterol-trioxolane. Cholesterol ozonolysis is very fast and complex, involving the decomposition of cholesterol-trioxolane into a series of unstable intermediates and end products [11,166,167]. Cholesterol-trioxolane rearrangement mainly results in reactive electrophiles (5,6-secosterol) which are in equilibrium with their carboxaldehyde, able to form adducts with proteins [174–176].

Molecular chlorine (Cl_2) can also oxidise cholesterol to generate a family of chlorinated oxysterols as for example α-chlorohydrin, β-chlorohydrin or 5,6-dichloro-cholesterol [167]. Molecular chlorine is generated by the phagosome myeloperoxidases in neutrophils during innate host defence. The bulky chlorohydrins affects host membrane stability and function and react with a variety of substances weakening thereby the invader cells.[177].

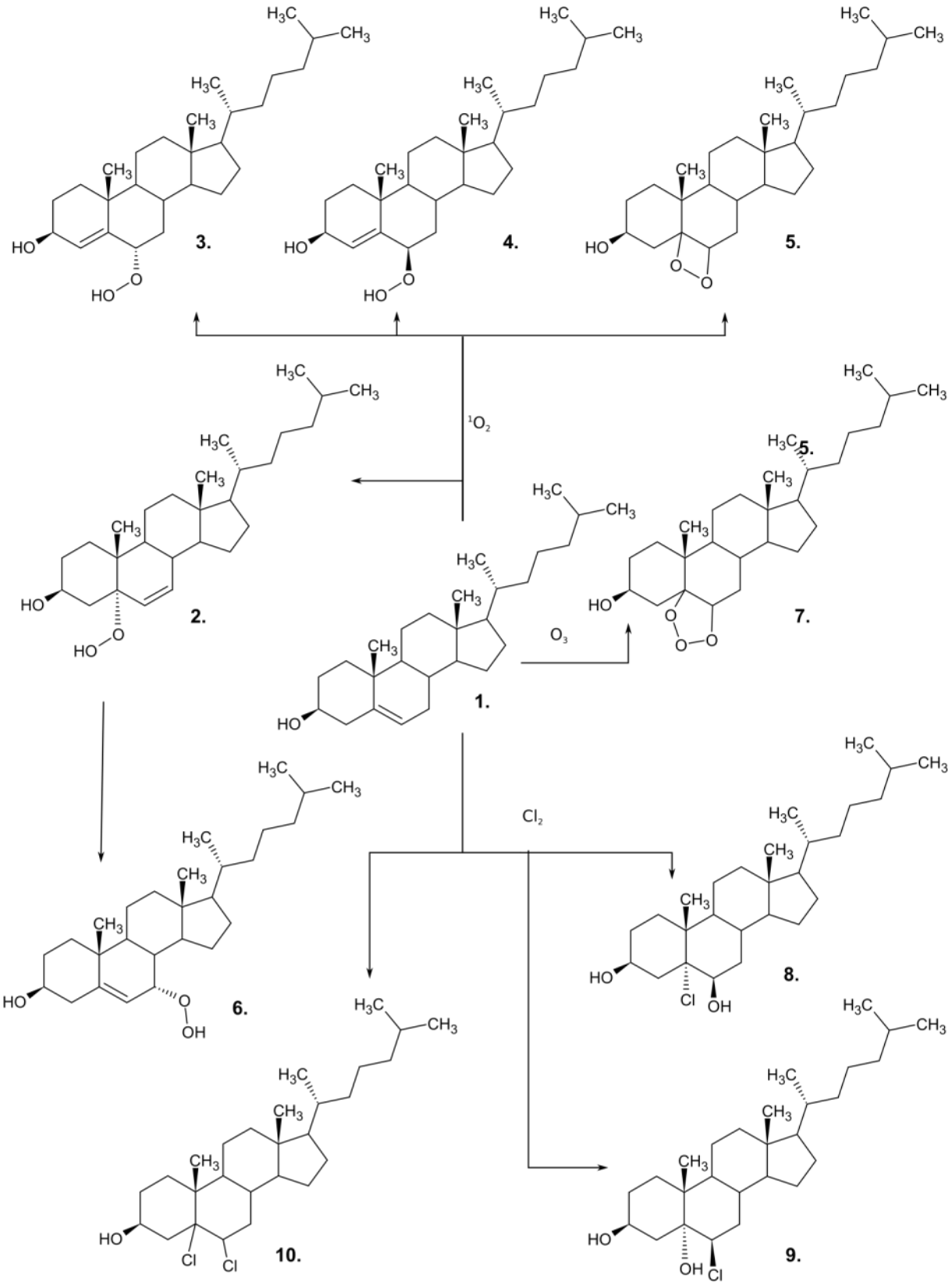

Figure 12| The non- radical mediated cholesterol oxidation pathway. Cholesterol (1.) is oxidised by singlet oxygen to result in four different oxysterol species: 5α-cholesterol-hydroperoxide (2.), 6α-cholesterol-hydroperoxide (3.), 6β-cholesterol-hydroperoxide (4.) and chol-dioxetane (5.). 5α-cholesterol-hydroperoxide (2.) can further react to result in 7α-cholesterol-hydroperoxide (6.). Ozone reacting with cholesterol (1.) leads to the unstable cholesterol-trioxolane (7.). Cholesterol reacting with molecular chlorine generates a family of chlorinated oxysterols like α- chlorohydrin (8.), β-chlorohydrin (9.) or 5,6-dichloro-cholesterol (10.) The abbreviations and acronyms are explained in the chapter. Figure adapted from Iuliano *et al.* (2011) [167].

1.1.6.1.2.3 OXYSTEROLS AS BIOMARKER FOR NON-ENZYMATIC MEDIATED CHOLESTEROL OXIDATION

H. Sies was the first to define oxidative stress as the imbalance between oxidants and antioxidants in favour of the oxidants, potentially leading to damage [178,179]. Basal to low level of oxidative stress is physiologically normal and controlled by antioxidant enzymes [180]. When the flux of ROS increases or the antioxidant is reduced, the organism is in intermediate to high oxidative stress condition and this raises modifications of different cellular components, considerably disturbing cellular homeostasis [180]. Because of the short life span of free radicals and their high reactivity, their measurement is limited to the detection of their end products [167]. The analysis of these resulting end products being the non-enzymatic mediated cholesterol oxidation products was considered to be specific and sensitive enough to be used as biomarker for oxidative stress [181,182]. L. Iuliano suggests the monitoring of free radical-mediated cholesterol oxidation by the mean of 7β-OHC, 7-KC, 5β,6β-epoxycholesterol, and cholestane-3β,5α,6β-triol (C-triol) levels [167] . For ozone/singlet oxygen-driven oxidation, as found for example in inflammation, 5,6-secosterol, 5α-hydroxycholesterol, and 6α-hydroxycholesterol could serve as biomarkers. In addition, Hazen *et al.* proposed the analysis of α- and β-chlorohydrins to assess for neutrophil-mediated tissue damage [183]. However, for the evaluation of oxysterols as new biomarker for a particular oxidative stress condition, it should be ensured that the potential biomarker is of real *in vivo* oxidation origin and not of *in vitro* origin occurring for example during sample preparation. In addition, as mentioned already, some oxysterols, as for example 7α-OHC, 4β-OHC and 25-OHC can be of both, enzymatic and of non-enzymatic origin [11,149,150]. The possible *in vitro* oxidation of cholesterol or oxysterols is an important pre-analytical issue influencing the measured oxysterols concentration.

1.1.6.1.3 ENZYMATIC CHOLESTEROL OXIDATION

Most *in vivo* oxysterols are of enzymatic origin and each oxysterol is synthetised by a different enzyme [1,152]. Enzymatic oxysterol biosynthesis was first discovered in 1956 by Frederickson and Ono using radiolabeled cholesterol incubation in subcellular fractions enriched in mitochondria, leading to the formation of 25-OHC and 27-OHC [16]. Almost all oxysterol synthetising enzymes belong to the class of monooxygenase of the cytochrome P450 (CYP) family which are named after the characteristic absorption wavelength at 450 nm [168]. This enzyme class consists of a disparate group of proteins with a similar structural fold containing in their active center a single heme-bound iron atom [184]. They are located in the membrane compartment of cells, either in the ER or in the mitochondria and utilise NADPH as a cofactor to catalyse oxysterol biosynthesis [149]. Cytochrome P450 monooxygenases differ in substrate specificity, sequence, tissue distribution and subcellular localisation [149,185].

In the following paragraphs the main enzymes of oxysterol synthesis are described in more detail and summarised in **Table 2.**

Table 2| Human enzymes implicated in oxysterol synthesis. The abbreviations and acronyms are explained in the chapter. Table adapted from Russell *et al.* (2000) [149].

Enyzme	Class	Subcellular localisation	Tissue mRNA levels	Substrate	Product	Enzyme commission number (EC), and References
Cholesterol 24-hydroxylase (CYP46A1)	Cytochrome P450	sER	Brain > liver	cholesterol	24(S)-OHC	1.14.13.98 [186]
Cholesterol 25-hydroxylase (CH25H)	Di-iron containing protein	ER, Golgi	Heart, lung, kidney, plus other tissues at low levels (interfollicular and outer follicular regions of lymphoid tissues)	cholesterol	25-OHC	1.14.99.38 [187]
Sterol 27-hydroxylase (CYP27A1)	Cytochrome P450	Inner mitochondrial membrane	Liver, small intestine, adrenal, lung, macrophages > kidney, spleen	cholesterol, cholesterol, lanosterol, zymosterol, desmosterol, 7-dehydrocholesterol, trihydroxycoprostan, 5β-cholestane-3α,7α,12α,27-tetrol,	27-OHC, 25-OHC, C27-diol and C27-acids of lanosterol, zymosterol, and desmosterol 27-hydroxy-7-dehydrocholsterol, 5β-Cholestane-3α,7α,12α,27-tetrol, 3α,7α,12α-Trihydroxy-5β-cholestanoic acid	1.14.13.15 [188,189]
Cholesterol 7α-hydroxylase (CYP7A1)	Cytochrome P450	sER	Liver	cholesterol	7α-OHC	1.14.13.17 [190]
Cytochrom P_{450} 3A4(CYP3A4)	Cytochrome P450	sER	Liver	cholesterol	4β-OHC	1.14.13. [157, 32, 67,97] [191,192]
2,3-Oxidosqualene cyclase (OSC)	Intra-molecular transferase	sER	Liver, macrophages	squalene monooxidosqualene	monooxidosqualene diepoxysqualene	5.4.99.7 [92,93,193]
Oxysterol 7α-hydroxylase (CYP7B1)	Cytochrome P450	ER	Brain, kidney, liver, reproductive tract, interfollicular and outer follicular regions of lymphoid tissues	25-OHC 27-OHC Dehydroepiandrosterone, Pregnenolone	7α,25-OHC 7α,27-OHC 7α-hydroxy Dehydroepiandrosterone 7α-hydroxy pregnenolone	1.14.13.100 [194–197]
Oxysterol 7α-hydroxylase 2 (CYP39A1)	Cytochrome P450	ER	Liver,	24(S)-OHC	7α,24(S)-OHC	1.14.13.99 [198]

1.1.6.1.3.1 STEROL 27-HYDROXYLASE

Sterol 27-hydroxylase (CYP27A1) is a mitochondrial cytochrome P450 oxidase distributed in a wide range of tissues and organs but is particularly highly expressed in liver, lung and in macrophages [1,168,199]. It is one of the rare cytochrome P450 enzymes of sterol side chain oxidation accepting different substrates and undertaking several reactions [200]. CYP27A1 depends on NADPH for its enzymatic activity and the availability of two electron transporters, adrenodoxin and adrenodoxin reductase [201]. Additionally, it is hypothesised that a specific mitochondrial membrane transporter is required to bring cholesterol to the enzyme [202]. The best-studied reaction is the first step of the "alternative" pathway of bile acid synthesis where cholesterol is converted to 27-OHC. 27-OHC is the major oxysterol found in human circulation and co-localises with macrophages in human atherosclerotic plaques [203,204]. Besides cholesterol also lanosterol, zymosterol, desmosterol, and 7-dehydrocholesterol can be used as substrate for the synthesis of oxysterols [189]. In addition to different substrates, the enzyme is also capable of sequential hydroxylation leading to the unexpected formation of 3β-hydroxy-5-cholestenoic acid [1]. CYP27A1 contributes, through the generation of 27-OHC, in two ways to cholesterol homeostasis: 27-OHC activates SREBPs, decreasing thus cholesterol synthesis. Furthermore, 27-OHC through binding to the LXRα receptor up-regulates the expressions of ABC transporters responsible for cholesterol cell efflux. Loss of function mutations in the CYP27A1 gene is known as Cerebrotendinous xanthomatosis (CTX) leading to cholesterol accumulation in the brain of CTX patients [205].

1.1.6.1.3.2 CHOLESTEROL 24-HYDROXYLASE

Cholesterol 24-hydroxylase (CYP46A1), is a brain-specific cytochrome P450 oxidase expressed in the smooth endoplasmic reticulum (sER) of neuronal cells which synthesise 24(S)-OHC [186,206]. 24(S)-OHC as blood-brain barrier passing molecule is found in the circulation before it is further metabolised to bile acids in the liver [21]. Since 24(S)-OHC represents the cholesterol efflux of the brain, it was proposed to be used as cerebral cholesterol turnover marker for neurodegenerative diseases such as Alzheimer disease [207–209]. In this particular disease, 24(S)-OHC increase in plasma is associated with decreased brain influx of blood-born Amyloid-β peptides characteristic of Alzheimer disease, hence, 24(S)-OHC was suggested to act as a brain protector [109,210,211].

1.1.6.1.3.3 CHOLESTEROL 25-HYDROXYLASE

25-hydroxycholesterol can be synthesised by autoxidation or by two different enzymes, either by CH25H or as a side product by CYP27A1 [11,187,212]. In comparison to all other oxysterol-synthetising enzymes, cholesterol 25-hydroxylase is not a member of the cytochrome P450 family but belongs to the non-heme iron-containing proteins [149,187]. This enzyme family appears also in bacteria and plants and they hydroxylate, desaturate, epoxidate or acetylinate various lipid substrates [149]. CH25H is located in the membrane of the ER and Golgi where its product, 25-OHC, exerts its function [186]. As mentioned before, 25-OHC is a regulator of the SREBP pathway for the transcriptional regulation of cholesterol homeostasis [149,187]. Although 25-OHC is a biologically important cholesterol metabolite it is only a minor oxysterol in human plasma [149].

1.1.6.1.3.4 OTHER OXYSTEROL SYNTHETISING ENZYMES

Although CYP27A1, CYP46A1, and CH25H have been extensively studied, there are many other oxysterol-synthetising enzymes. For example cholesterol 7α-hydroxylase is an important hepatic enzyme producing 7α-OHC in the first step of the "classical" bile acid pathway [213]. CYP3A4 is a drug-metabolizing enzyme converting cholesterol into 4β-OHC [150,214]. 24(S),25-EC is produced by 2,3-oxidosqualene cyclase in a shunt of the same mevalonate pathway that produces cholesterol [94]. The enzymatic synthesis of dihydroxycholesterols like 7α,25-OHC or 7α,27-OHC occurs in a 2 step manner: first the corresponding side-chain mono-hydroxycholesterols are synthesised followed by a C-7 hydroxylation by CYP7B1 [197,215,216]. An exception occurs for 7α,24(S)-

dihydroxycholesterol (7α,24(S)-OHC), for which the second hydroxylation is mediated by CYP39A1 instead of CYP7B1 [217]. In the case of 7α,25-OHC it is known that both required enzymes, CH25H and CYP7B1 are expressed in the ER of for instance lymphoid stromal cells, indicating a straightforward synthesis pathway of dihydroxycholesterols at the location of their function [35,130].

The number of enzymes implicated in oxysterol synthesis indicates that oxysterol formation is a tightly regulated process in many vertebrates in order to trigger specific signalling pathways of for example the immune system and to precisely control the cell and body cholesterol concentration [34,149,218].

1.1.6.2 CELLULAR LEVELS AND LOCATION

Oxysterols are like cholesterol, confined either in non-polar cell locations meaning in cell membranes, lipid droplets or in the circulation in low density lipoproteins (LDL) [1,168]. Under normal physiological conditions, their concentrations are about 10^3 to 10^6 times lower than the concentration of cholesterol [1,31]. *In vivo* studies on subcellular localisation are rare and most quantification attempts were carried out in human plasma **(Table 3)**. The concentration of each oxysterol in plasma varies but generally the order of magnitude for free oxysterols is around 5-50 ng/mL. Most plasma quantification studies are made on free oxysterols being biologically active but on a cellular level most enzymatic derived oxysterols are inactivated through esterification to approximately 74-98 % [219–221]. The situation in plasma clearly shows that oxysterols do not occur alone but always in a mix of different species, making their individual study difficult [1].

Table 3| oxysterol plasma concentrations reported in the literature. All values have been converted into nM allowing a simpler comparison. The abbreviations and acronyms are explained in the chapter.

Compound	Concentration of free OSt in ng/mL (nM)	Concentration of total OSt in ng/mL (nM)	Co-elution of 24(S)-OHC and 25-OHC
24S-OHC	5,46 ± 0,05 (13,56 ± 0,12) [222] 5,46 ± 0,19 (13,56 ± 0,47) [223] 6.86 ± 0,31 (17,04± 0,77) [224]	22,1 ± 0,8 (54,9 ± 10,2) [223] 51 ± 12 (127 ± 30) [225] 56,1 ± 1,18 (139.3 ± 2,93) [226] 64 ± 14 (159 ± 44) [227] 146 ± 6 (363 ± 15) [156]	1,77 ± 0,6 (4,4± 1,5) [220]
25-OHC	0,7 ± 0,02 (1,74 ± 0,05) [222] 4,06 ± 0,22 (10,08 ± 0,55) [224]	9 ± 1 (22± 2) [156] 11,8 ± 0,28 (28,3 ± 0,70) [226] 31 ± 11 (77 ± 27) [225]	
27-OHC	10,33 ± 0,60 (25,65 ± 1,49) [222] 15,52 ± 0,96 (38,54 ± 2,38) [220] 19,12 ± 70,70 (47,49 ± 175,59) [224]	117 ± 35 (291 ± 87) [225] 120 ± 30 (298 ± 75) [227] 151,4 ± 3,03 (376 ± 7,5) [226] 235 ± 3 (628 ± 7) [156]	
7-KC	0,59 ± 0,33 (1,47 ± 0,82) [222] 5,51 ± 3,16 (13,68 ± 7,85) [223] 22,8 ± 3, 0 (56,6 ± 7,5) [228] 77,4 (192,2) [26]	49,1 ± 33,4 (121,9 ± 82,9) [223] 84 ± 3,78 (209 ± 9,39) [226]	
7α25-OHC	<0,2 (<0,48) [224] 0,09 ± 0,02 (0,21 ± 0,05) [220] 0,12 ± 0,04 (0,29 ±) [222]		
7α27-OHC	0,26 ± 0,09 (0,62 ± 0,22) [222] 1,42 ± 0,04 (3,4±0,1) [220] 1,65 ± 0,39 (3,94 ± 0,97) [224]	10 ± 0,24 (23,89 ± 0,60) [226]	
C-triol	9,7 ± 1,2 (24,1 ±3,0) [228] 20,1 (49,9) [26]		

1.1.6.3 OXYSTEROL METABOLISM

Oxysterols can be further metabolised in the cell by four mechanisms often requiring the same enzyme set as cholesterol does **(Figure 13)**:

1. Esterification of oxysterols is mediated (like for cholesterol) by acetyl-coenzyme A-acetyltransferase in cells and lecithin-cholesterol acyltransferase in plasma. Acetyl-coenzymeA -acetyltransferase possesses two sterol-binding sites: one being catalytic the other being an allosteric activator. Interestingly only cholesterol is able to bind strongly to the allosteric site, meaning that oxysterols need cholesterol for their own esterification, which reflects a possible *in vivo* scenario. Like for cholesterol the esterification biologically inactivates the oxysterols [152,219,221,229].

2. Some enzymes like CYP27A1 are able to act on cholesterol and on few ring-oxygenated sterols leading to further oxidation products. Since this secondary oxysterols are more hydrophilic they can be easily excreted from cells and are therefore associated with less cellular toxicity than the parent oxysterol [230–232].

3. Sulphation may occur by sulphotransferases (SULTs) like SULT2 specifically sulfating the 3β-hydroxyl group of steroids and sterols or SULT2B1b sulphating ring-oxygenated side-chain oxysterols. The oxysterol- sulphate conjugation resulting into more hydrophilic compounds, is an important cell detoxification mechanism for non-enzymatic derived oxysterols [233]. In addition, oxysterol sulphation inactivates the oxysterol activity on LXRs [234]. Cholesterol sulphation products are present in all mammalian cells but are particularly abundant in keratinised tissue, such as skin and hooves. Cholesterol sulphate, the main circulating sterol sulphate in plasma is supposed to have a role in skin layer integrity and adhesion [24,168,233,235].

4. A reduction reaction was discovered for 7-KC to obtain 7β-OHC by 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD 1). This metabolisation step is suggested to be a detoxifying mechanism of dietary 7-KC in the liver and in adipose tissue [236]. 11β-HSD 1 main enzymatic function is, however, the conversion of cortisone to the active receptor-binding derivative cortisol in glucocorticoid metabolism, thus indication a possible interconnection between oxysterol and glucocorticoid metabolic pathways [236,237].

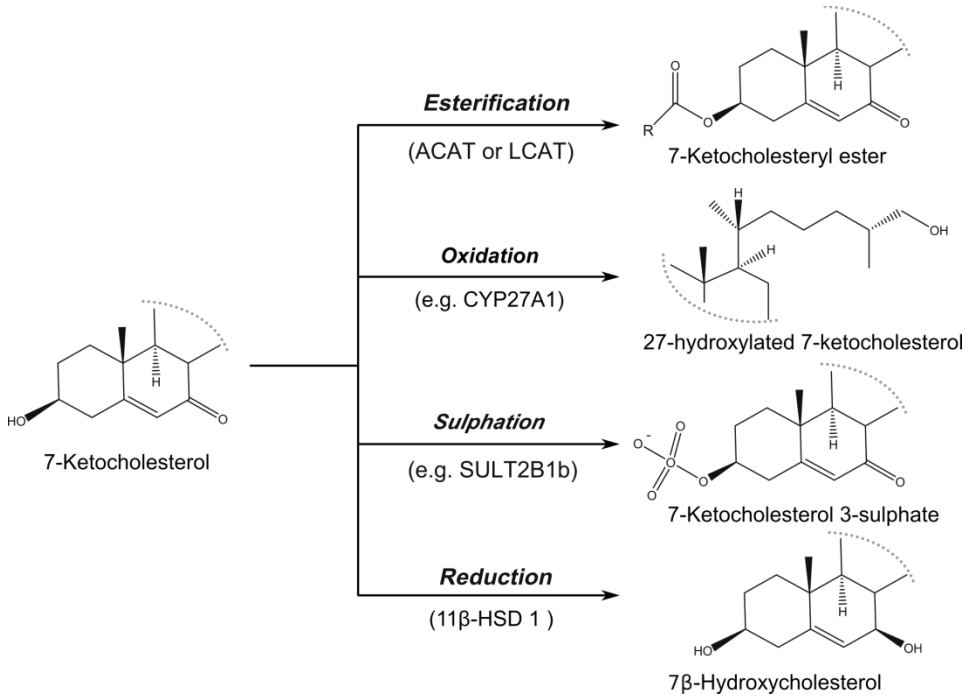

Figure 13| Possible reactions occurring on oxysterols. Oxysterols can be esterified, oxidised, sulphurised and reduced. The abbreviations and acronyms are explained in the chapter. Figure adapted from Brown *et al.* (2009) [1].

1.1.6.4 OXYSTEROL ELIMINATION

As previously discussed, oxysterol are confined in non-polar locations due to their hydrophobic nature. This is the reason why they cannot be directly eliminated from cells apart from a few exceptions like 24(S)-OHC or 27-OHC having a more hydrophilic side-chain [238]. The elimination process requires therefore specific membrane lipid transporters and plasma carriers. Examples of such transporters are scavenger receptor B1, ABCA1 or ABCG1 [239]. ABCA1 is a phospholipid flippase that transports phospholipids and cholesterol to lipid-free ApoA1. Through its flippase activity, ABCA1 changes the lipid packing of the plasma membrane. ApoA1 is the major protein component of high-density lipoproteins and contains multiple amphipathic α-helical domains. It is suggested that ApoA1 interacts via its hydrophobic faces with the plasma membrane directly after ABCA1 flippase activity. Once ApoA1 is bound to the plasma membrane it desorbs some membrane phospholipids, cholesterol and oxysterols [1]. A second possible oxysterol elimination pathway is through passive diffusion that can occur spontaneously for cholesterol or oxysterols from cell membranes to HDL [240,241]. The diffusion rate of 7-KC and 7β-OHC to the HDL particle was found to be similar as for cholesterol [240]. Passive diffusion can be accelerated by proteins like scavenger receptor B1 able to make a bi-directional exchange or by ABCG1 capable of a uni-directional export to the HDL particle [239]. It is hypothesised that ABCG1 promotes the efflux of toxic oxysterols like 7-KC and 7β-OHC, which are considered to induce apoptosis in cells, thereby protecting the cell.

1.1.7 TOXICITY AND PATHOLOGICAL EFFECTS OF OXYSTEROLS

Oxysterol toxicity was characterised early and was mainly interpreted in 1981 and 1989 by Smith as a "biological effect" [11,32]. At that time, he already exhaustively summarised the aspects of cytotoxicity, atherogenicity, mutagenicity, carcinogenicity and their effect on cell membranes. Nevertheless, he was not aware of how exactly oxysterols lead to these effects and even nowadays not all oxysterol toxicological properties are completely understood. Oxysterol toxicity and side effects can trigger pathophysiological conditions, such as cancer, atherosclerosis and neurological disorders, and probably many more. However, conclusions on *in vivo* oxysterol toxicity have to be taken cautiously since healthy cells have mechanisms to eliminate or store excess oxysterols. **Figure 14** is a summary of the described toxic and pathological effects of oxysterol *in vivo*.

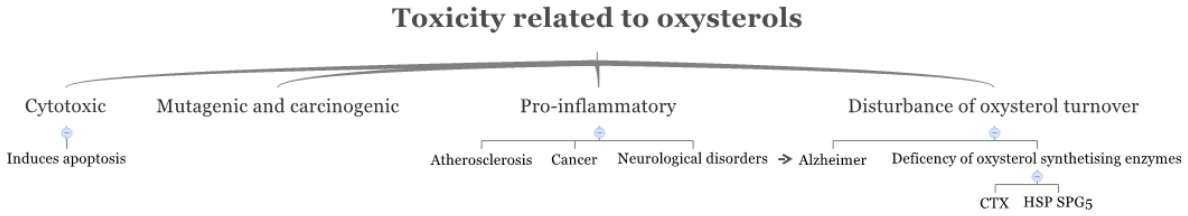

Figure 14| Summary of the toxicity attributed to oxysterols. They are considered to have cytotoxic effects, mutagenic and carcinogenic properties, are pro-inflammatory and a disturbance in their own turnover can have negative health effects. The abbreviations and acronyms are explained in the chapter.

1.1.7.1 CYTOTOXIC PROPERTIES OF OXYSTEROLS

Oxysterol cytotoxicity was studied *in vitro* in different cell types as well as *in vivo* in different animal models [31,32,242,243]. The observed cellular effects after *in vitro* oxysterol administration were diminished cellular functions, with reduced cellular growth, and reduced cellular proliferation. Similar observations were made *in vivo* with cessation of growth, loss of weight, and diminished appetite, in addition to damages to arteries and increased aortal lesion formation in models of atherosclerosis [32]. Cytotoxicity of the most cytotoxic oxysterols (7-hydroxy-, 7-keto- and triol cholesterol derivatives) is thought to be due to perturbation of the intracellular calcium levels, as well as the overproduction of intracellular ROS affecting membrane fluidity and integrity [243,244]. The cellular consequence of oxysterol cytotoxicity is apoptosis, which is the desired effect in cancer

therapy making oxysterols an interesting drug candidate for treatment [245]. In contrary, Mathieu *et al.* were interested to increase cellular resistance towards the cytotoxic activity of oxysterols by lysosomal-targeted clearance of 7-KC. The reasoning behind it is that cellular sterol uptake occurs via receptor-mediated endocytosis of LDL leading to sterol accumulation in lysosomes. Consequently lysosomes are a major site of non-enzymatic oxysterol concentration and Brown *et al.* could show that the 7-KC level is particularly high in endosomal and lysosomal compartments [232]. However, 7-KC-metabolising enzymes are localised in other organelles as for example in the ER, in the mitochondria and in the cytoplasma. With the lysosomal-targeted *Chromobacterium sp*. DS1 cholesterol oxidase, Mathieu *et al.* were able to enhance fibroblast cell resistance towards 7-KC thus being an interesting therapeutic approach for disorders accumulating non-enzymatic oxysterols [246].

1.1.7.2 MUTAGENIC AND CARCINOGENIC PROPERTIES OF OXYSTEROLS

In contradiction to the reported cytotoxic effects, oxysterols were also described to be mutagenic and carcinogenic. Oxysterol mediated mutagenicity was only found for C-triol, and 7-KC on Chinese hamster ovary epithelial cells, and on mitochondrial DNA of retinal pigmented epithelium cells, respectively. This led Gramajo *et al.* to propose oxysterol mutagenicity to be implicated in the pathogenesis of retinal diseases [247,248].

In addition to oxysterol mutagenicity, different cancers where shown to be either initiated or further progressed by oxysterols as it is for example the case in cholangiocarcinogenesis by 22*R*-hydroxycholesterol. In this particular situation, 22*R*-hydroxycholesterol stabilises cyclooxygenase-2 (COX-2) mRNA via the p38-Mitogen-activated protein kinase pathway enhancing thus COX-2 protein expression. Cyclooxygenase-2 promotes cellular replication and inhibits apoptosis contributing so to cholangiocarcinogenesis progression [249–251].

1.1.7.3 ATHEROSCLEROSIS AND OXYSTEROLS

Atherosclerosis is a chronic inflammatory disease closely related with the deregulation of lipid metabolism [252]. It is a pathological process consisting in an inflammatory signal cascade leading to lipid and cell accumulation in the intimal arterial wall resulting in plaque formation [253]. Atherosclerosis can be considered as a three-step process consisting in lesion initiation, lesion progression and plaque rupture. The lesion initiation is due to hyperlipidaemia in arterial walls, which activates endothelial cells for rapid monocyte influx into the arterial intima. In the arterial intimia, the monocytes-derived macrophages take up oxidised LDL through their scavenger receptors giving thereby rise to foam cells, visible as fatty streaks. If the influx is persistent, we come to atherosclerosis second step of lesion progression in which cytokines and growth factors secreted from leukocytes promote lymphocyte and smooth muscle cell migration. The migrated smooth muscle cells as well as their released matrix proteins form a fibrotic cap blocking off the foam cells [252,254]. Finally, plaque disruption is accelerated by the apoptosis of foam cells releasing metalloproteinases (MMP) which degrade the fibrotic cap and thus destabilise it [254]. Disrupted plaques lead to thrombus formation which blocks the arterial blood cirulation leading to heart attacks and ischemic strokes [255]. It was discovered that oxysterols, mainly 27-OHC is produced in atherosclerotic lesions via CYP27A1 in macrophage-derived foam cells [152,199,204,256]. The oxysterol production in atherosclerosis was considered to be a defence response towards the high cholesterol load [204]. However recently, Umetani *et al.* identified 27-OHC as ligand of the estrogen receptor α (ERα) which surprisingly triggers an upregulation of pro-inflammatory genes in contrary to estrogen [257,258]. The pro-inflammatory action is mediated through Nuclear factor-κβ (NF-κβ) activation via the extracellular-signal-regulated kinases 1 and 2 (Erk1,2) and c-Jun N-terminal kinase (JNK)-dependent degradation of the nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα). From these results, the authors conclude that 27-OHC promotes atherosclerosis via activation of pro-inflammatory pathways as well as via the attenuation of estrogen-related atheroprotection [257]. In addition other oxysterols, like 25-OHC stimulate IL-8 production, which is an important cytokine of atherosclerosis since it recruits neutrophils and vascular smooth muscle cells into the subendothelium. IL-8 also inhibits the expression of the local inhibitors of

metalloproteinases-1 (TIMP-1), thus enhancing fibrous cap destabilisation [259–261]. This finding demonstrates that oxysterol mediated IL-8 production establishes and preserves the inflammatory micro-environment in arterial cell walls and contributes to plaque disruption.

1.1.7.4 NEUROTOXICITY OF OXYSTEROLS

Mutations in oxysterol-forming enzymes, oxysterol excess, as well as depletion can have neurological consequences, as it is the case for Alzheimer disease, CTX and for Hereditary Spastic Paresis (HSP) type SPG5 [202]. Oxysterol's implication in Alzheimer disease was already mentioned and will not be further discussed here, while CTX was only shortly mentioned. CTX is a rare inherited autosomal recessive disorder caused by mutations in the CYP27A1 gene with the translated enzyme located at the inner mitochondrial membrane in most cells of the organism [202]. Due to CYP27A1 mutations, CTX patients have a reduced rate of bile acid synthesis, and therefore the normal homeostatic feedback regulation of cholesterol is disrupted, which further enhances the production of bile alcohols and cholestanol from bile acid precursors. The consequence is the formation of xanthomas potentially anywhere in the body but particularly in the nervous system, in atherosclerotic plaques and tendons of CTX patients. The most serious disease effect arises due to the xanthomas in the neurological system, which preferentially occurs in the brain white matter. CTX patients are characterised by dementia, ataxia and cataracts. Typical disease onset is in infancy with cholestatic jaundice, cataracts and diarrhea followed by mental retardation. Untreated CTX patients usually die between the ages of 30 years and 60 years from progressive neurological dysfunction or myocardial infarction [262].

The Hereditary Spastic Paresis are a genetically and clinically heterogeneous group of upper-motor-neuron degenerative diseases characterised by selective axonal loss in the corticospinal tracts and dorsal columns [263]. Symptoms include progressive spasticity of the lower limbs and muscle weakness, frequently associated with deep sensory loss and urinary urgency. In addition more "complex" Hereditary Spastic Paresis are accompanied by other neurological signs such as ataxia, mental retardation, dementia, visual dysfunction, and epilepsy [202]. Recently, about 40 different loci for Hereditary Spastic Paresis were identified with various types of inheritance [264–266]. The first locus defined was named SPG5 and was later identified to be the CYP7B1 encoding gene [263]. CYP7B1 has broad substrate specificity including 25-OHC, 27-OHC, and a number of C19 and C21-steroids [194–197]. CYP7B1 is of importance both for bile acid synthesis, which is a well-understood role, and for the metabolism of neurosteroids, much less studied. When CYP7B1 is mutated, monohydroxycholesterols can't be further metabolised and therefore Hereditary Spastic Paresis patients with the SPG5 mutation, have increased plasma levels of 27-OHC (6- to 9-fold) and 25-OHC (100-fold) [267].

1.1.8 STRATEGIES FOR OXYSTEROL ANALYSIS

Despite oxysterol's early discovery over one century ago, their research was hampered by the challenges of accurate separation-, detection- and identification methods. Most discoveries were made possible in the last 20 years due to the improved analytical instrumentation. The challenge of oxysterol analysis is to overcome several parallel difficulties, namely:

1. Cholesterol presence in large excess
2. Low cellular concentrations
3. Autoxidation of cholesterol and oxysterols
4. Cellular localisation in membranes
5. Possible chemical modifications like esterification, reduction, or sulphation
6. Chemical structures and properties [1,24,31,148,152,233,268,269].

The last issue is an important since oxysterols do not possess a strong chromophore making them difficult or impossible to detect by ultraviolet absorption (UV)-spectroscopy. Furthermore, oxysterols are neutral hydrophobic molecules, poorly ionisable in electrospray (ESI) and atmospheric pressure chemical ionisation (APCI) and are mainly isomeric compounds with very similar mass spectrometry (MS) spectra. The different existing methods for analysis will be discussed in the next section **(Figure 15)**.

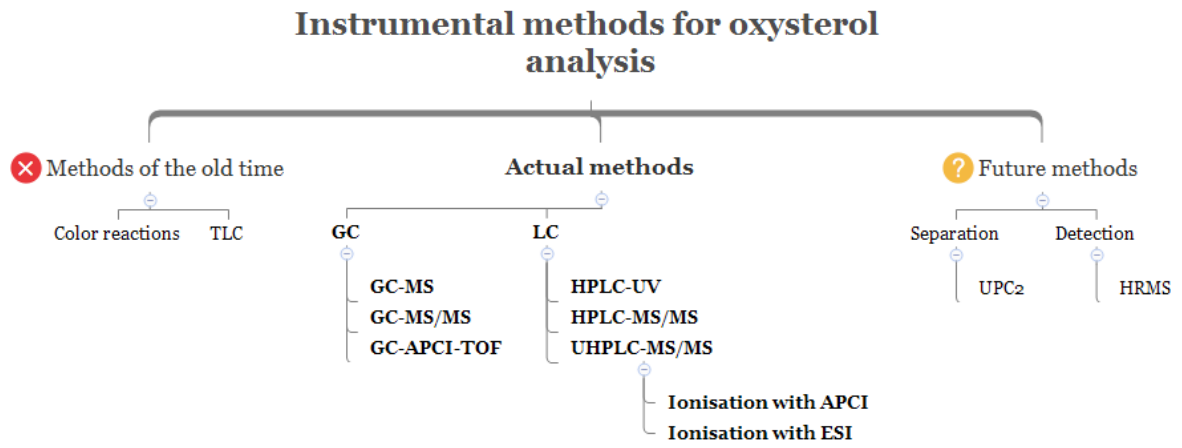

Figure 15| Summary of the instrumental methods used in the past, currently and probably in the future to identify and quantify oxysterols in biological matrices. The abbreviations and acronyms are explained in the chapter.

Lifschütz, the discoverer of cholesterol oxidation products detected oxysterols using UV-vis spectrophotometry after a colour-inducing reaction was carried-out with highly concentrated sulphuric acid [9]. Since it neither allows separation nor identification of oxysterols with similar structure, this detection method fell into oblivion. Thin layer chromatography (TLC) has the advantage of being cheap, simple and rapid and was used as a method for oxysterol analysis in the 70th and 80th. The strategy was the detection of oxysterols trimethylsilyl (TMS) ether derivatives or Δ^4-3-keto derivatives (Δ= position of the double bond) obtained by enzymatic transformation with cholesterol oxidase. The thin layer chromatography stationary phase consisted in silica, silica gel, silica-G60 or silica gel G-AgNO3 plates with or without fluorescent indicator, mainly separating mono-hydroxycholesterols from each other [31]. Aringer and Nordström did an impressive task in separating by thin layer chromatography, GC-MS and in the combination of both the standards of 165 oxygenated sterols including oxygenated derivatives of plant sterols [270]. As highlighted by Schroepfer, also this large collection of C-27 oxysterols did not include all physiologically relevant oxysterols with oxygen functions at carbon atoms 4, 20, and 22 and 24,25-epoxysterols [31]. This study proved that thin layer chromatography has a limited capability to provide useful oxysterol separation in complex mixtures such as blood and tissues.

Separation of complex mixtures is best archived on LC, but usually LC was coupled to a ultraviolet–visible detector (LC-UV/VIS). As mentioned before, oxysterols UV-absorption is very limited, making their analysis not suitable for LC-UV/VIS. Nevertheless, Teng and Smith were able to separate and detect 6 different oxysterols (7β-OHC, 7-KC, 19-OHC, 20-OHC, 25-OHC, 26-OHC) with LC-UV-vis, after enzymatic transformation by cholesterol oxidase converting 3β-hydroxy-5-ene groups to UV-absorbing (231–233 nm) 3-oxo-4-enes [271]. Zhang exploited this method for 24(S)-OHC, 25-OHC and 27-OHC analysis in rodent liver [272].

1.1.8.1 CURRENT STRATEGIES FOR OXYSTEROL ANALYSIS

1.1.8.1.1 GC-MS

In 1995, Dzeletovic *et al*. published an oxysterol GC-MS (EI) method for the analysis of human plasma that became the reference method [156]. Several groups applied it to determine oxysterol levels in tissues, cells and cerebrospinal fluid, to extend the analyte panel or to further optimise it [273–279]. Griffith *et al.* summarised the key steps of Dzeletovic's method as follow:

> "1. Blood was collected in Ethylenediaminetetraacetic acid (EDTA) K_3 containing vacutainers, and following centrifugation (1400 g, 10 min) to prepare plasma, butylated hydroxytoluene (BHT, 50 mg/mL) was added. BHT is an antioxidant while EDTA traps metal ions there-by avoiding the Fenton reaction and metal catalysed oxidations. Deuterium-labelled internal standards (IS) were added at this point.
>
> 2. Alkaline hydrolysis was performed under Ar using 0.35 M ethanolic potassium hydroxide (KOH) for 2 h at room temperature (22 °C). The solution was neutralised with phosphoric acid and sterols were extracted into chloroform. The solvent was evaporated and the residue was dissolved in toluene.
>
> 3. Oxysterols were separated from cholesterol on a silica cartridge, cholesterol eluting in 0.5% propan-2-ol in hexane followed by oxysterols in 30% propan-2-ol in hexane.
>
> 4. After removal of solvent, samples were converted to trimethylsilyl ethers by treatment with 350 mL of pyridine:hexamethyldisilylazane:trimethylchlorosilane (3:2:1, v/v/v) at 60 °C for 30 min, dried down and re-dissolved in 100 mL of hexane **.**
>
> 5. GC–MS analysis was performed utilising selected ion monitoring (SIM) to achieve maximum sensitivity [222]."

To our knowledge, the Schmitz Group made the latest improvement of oxysterol separation with gas chromatography. They published two new methods: one based on GC-MS/MS, the other on gas chromatography coupled to time of flight mass spectrometry (GC-TOF) [280,281]. The 8.5 min long GC-MS/MS method was used for the simultaneous analysis of 24(S)-OHC, 25-OHC, 27-OHC, 7-KC, lanosterol, lathosterol, 7-dehydrocholesterol, desmosterol, stigmasterol, sitosterol and campesterol. For this, they hydrolysed the molecules with ethanolic potassium hydroxide solution, extracted them by liquid/liquid extraction with n-hexane, derivatised them with N-methyl-N-trimethylsilyl-trifluoracetamide and applied positive chemical ionisation with ammonia, as reagent gas. Tandem MS allowed the use of specific multiple reaction monitoring (MRM) transitions, which allowed separating of co-eluting substances, like desmosterol and 7-dehydrocholesterol, sitosterol and lanosterol, or lathosterol and zymosterol. The oxysterol experience gained through the GC-MS/MS method served as basis for a GC-TOF-MS method, in which the purpose was the proof of principle for the method and not the separation itself. The author's aim was to combine the excellent peak resolution of GC with the soft ionisation of APCI ionisation sources and the high resolution obtained by TOF. Although the first attempt of coupling GC with APCI were made by Horning *et al*. in the 1970s and there are now commercially available sources from different manufacturers, GC–APCI–TOF-MS has not widely spread [282,283]. After modification and optimisation of a commercial APCI interface, they applied the GC–APCI–TOF-MS method to the separation and detection of 7β-OHC, 27-OHC, 24(S)-OHC, 7-KC, lanosterol, lathosterol, sitosterol, and campesterol. The results were satisfying and the authors claimed it to be attractive for the identification of unknown and unexpected components but didn't further comment on it [281].

1.1.8.1.2 LIQUID CHROMATOGRAPHY COUPLED TO TANDEM MS (LC-MS/MS)

Although GC-MS has the advantage of showing excellent chromatographic resolution, being easy to use and having relatively low cost for instrumentation run, it has limitations. Oxysterol analytics on GC-MS is mainly hindered by: (i) labor-intensive sample preparation, (ii) high injection volume, (iii) lack of sensitivity requiring relatively large plasma volumes, (iv) mass spectral scanning functions and (v) the loss of derivatising groups in the ion source making molecular weight determination difficult [226,284,285]. High-performance liquid

chromatography–tandem mass spectrometry (HPLC–MS/MS) and ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC –MS/MS) even more, are powerful tools for the microanalysis of compounds in biological samples. Compared to GC-MS methods, they are more broadly applicable to various analytes especially thermo- sensitive ones, they are more versatile and flexible for stationary phase change and don't suffer from baseline drift as much as GC column do. Sufficient selectivity is achieved by the combination of chromatographic separation and selected reaction monitoring [286]. According to Ayciriex *et al.* LC-MS is nowadays the most frequently used technique for the identification and quantification of sterols and oxysterols in complex biological samples [284]. However, also LC-MS/MS has its limitations for oxysterol analysis. One issue is that oxysterols are isomeric compounds resulting in very similar spectra and putting thereby a high demand on chromatographic performance. Identification of unknowns is almost impossible without authentic standards. The second issue is the poor ionisation capacity of oxysterols in both ESI and APCI ionisation sources, motivating many groups to oxysterols derivatisation prior LC-MS/MS analysis [222].

1.1.8.2 OXYSTEROL ANALYSIS IMPROVEMENT

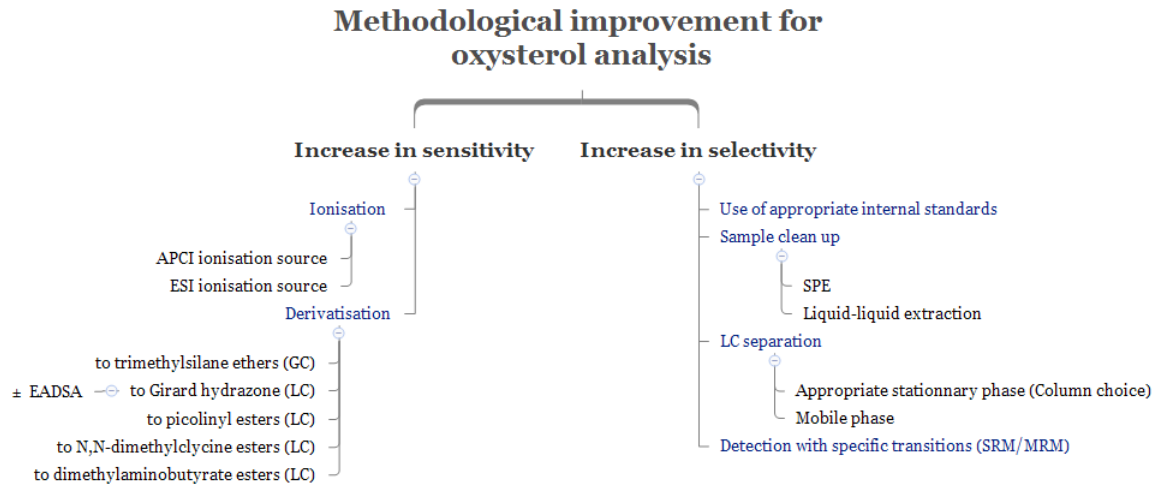

Figure 16| Diagram of possible methodological improvements for oxysterols analysis. The abbreviations and acronyms are explained in the chapter.

1.1.8.2.1 SENSITIVITY IMPROVEMENT FOR OXYSTEROL ANALYSIS.

1.1.8.2.1.1 IONISATION

Standard ionisation sources for LC-MS/MS analysis are ESI and APCI, which are often exchangeable in the same mass spectrometer. In ESI the "sample solution is sprayed across a high potential difference (a few kV) from a needle into an orifice in the interface. Heat and gas flows are used to desolve the ions existing in the sample solution" [287]. Although the principle is quite simple there is still no consensus on the theory that explains the final production of gas-phase ions. ESI is a soft ionisation technique that is broadly applicable for polar compounds and a large range of molecular weights. Limitations of ESI are the generation of multiply charged species making the interpretation more complex than for other ionisation sources and the bad ionisation of uncharged, non-basic and low- polarity compounds like steroids. This is the reason why this compound class including oxysterols was preferentially analysed with the complementary method to ESI, namely APCI. APCI is also a soft ionisation method but in which "a corona discharge is used to ionise the analyte in the atmospheric pressure region. The gas-phase ionisation in APCI is more effective than ESI for analysing less-polar species" and was considered to be sensitive enough for direct analysis of sterols without derivatisation [288–291]. APCI's

inconvenience is the dehydration of protonated molecules hindering often the identification of the analyte's molecular weight [291,292]. Oxysterol detection limits obtained with HPLC-APCI–MS are reported to lie in the range of 0.1–0.8 ng (about 0.25–2.0 pmol) [293,294]. In 2007 McDonald *et al.* showed efficient ionisation of neutral sterols by HPLC–ESI–MS/MS using the Applied Biosystems 4000 QTrap triple quadrupole system [295]. The authors reported on-column detection limits of 5–20 fmol and 25–2000 fmol for dihydroxy- or epoxysterols, and monohydroxycholesterols, respectively [295]. These values are not inferior to those obtained by APCI but still three orders of magnitude higher than for examples oxysterols derivatised with picolinoyl esters [286,296]. However, it is difficult to make a general statement on ESI sensitivity since it is known to be strongly instrument-dependent [286].

Independent of the ionisation method used, oxysterol ions are often adduct $[M+NH_4]^+$ ions that result in very few fragmentation product ions arising from carbon-carbon bond cleavages. Oxysterols form mainly a 369 m/z fragment due to the elimination of one or two neutral water molecules, or analogue ions depending on the number of hydroxyl groups and the basic steroid structure. The stability of the cyclic ring structure confers resistance towards ionisation and further fragmentation (MS/MS) at higher energies thus giving very little structural information of simple mono-and dihydroxycholesterols [297].

Improved MS sensitivity, however, allowed several groups to go for underivatised oxysterol analysis avoiding the laborious sample preparation. The main chosen ionisation method is APCI. Besides McDonald *et al.* only Karuna *et al*. published an ESI-MS/MS oxysterol method of underivatised oxysterols. The group was able to separate 7β,25-OHC, 7β,27-OHC, 7α,25-OHC, 7α,24S-OHC, and 7α,27-OHC and quantified the low concentrated dihydroxycholesterols, 7α,25-OHC and 7α,27-OHC in human plasma while having a limit of detection (LOD) of 0.5 nM [220]. According to their experience, the ionisation efficiency for dihydroxycholesterols decreased in the following order: ESI > APCI > assisted-atmospheric pressure photoionisation, contradicting thereby an old ionisation dogma.

1.1.8.2.1.2 DERIVATISATION

To circumvent unspecific oxysterol fragmentation and bad ionisation, a set of different derivatisation methods were proposed **(Figure 17 and Figure 18)**.

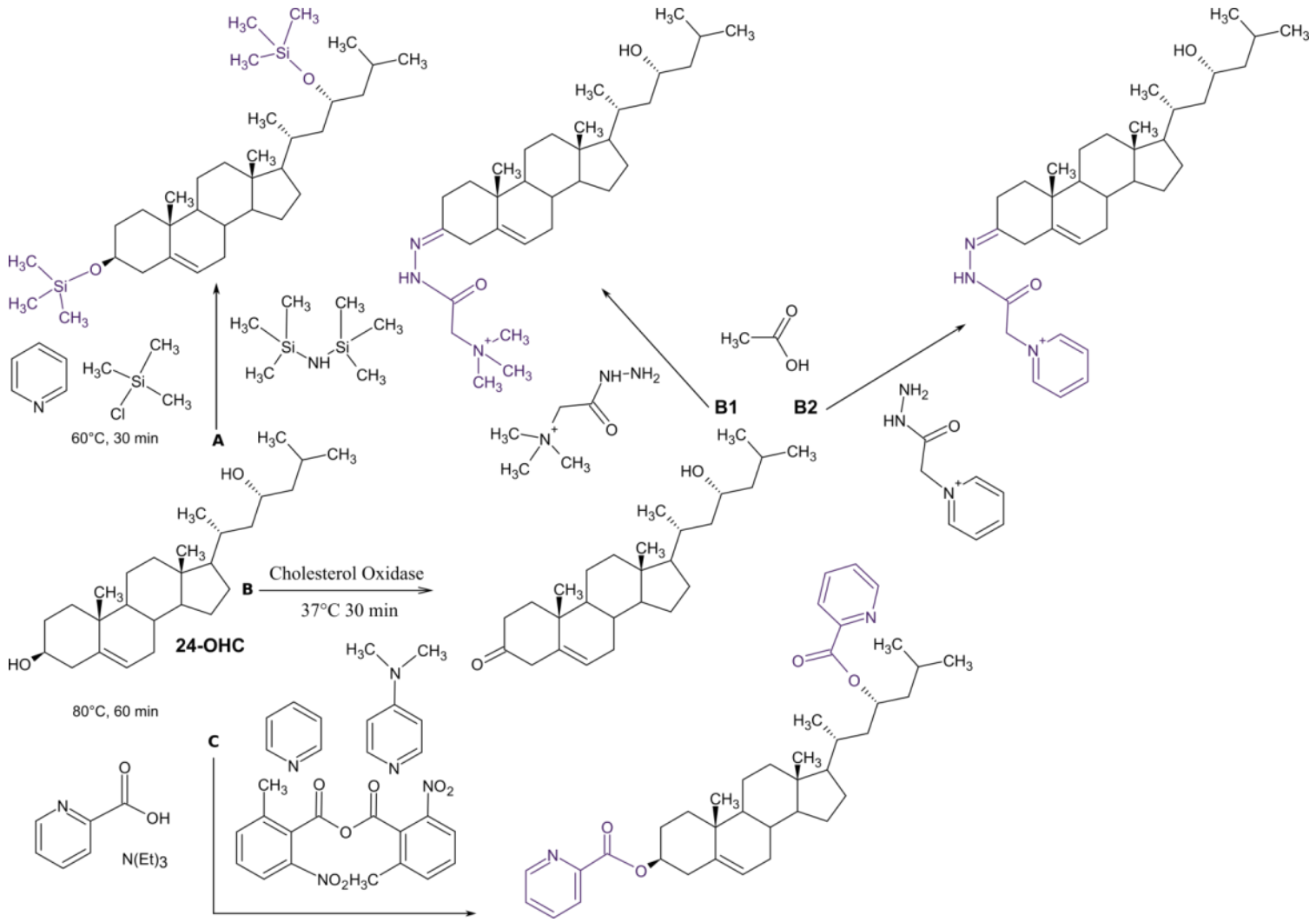

Figure 17| Oxysterol derivatisation reactions (Part 1) A. Derivatisation to trimethylsilyl ethers for GC-MS analysis B. Derivatisation to Girard hydrazones with Girard T (B.1) or Girard P reagent (B.2) C. Derivatisation to picolinyl esters . The abbreviations and acronyms are explained in the chapter. Figure adapted from Griffiths *et al.* (2013) [222].

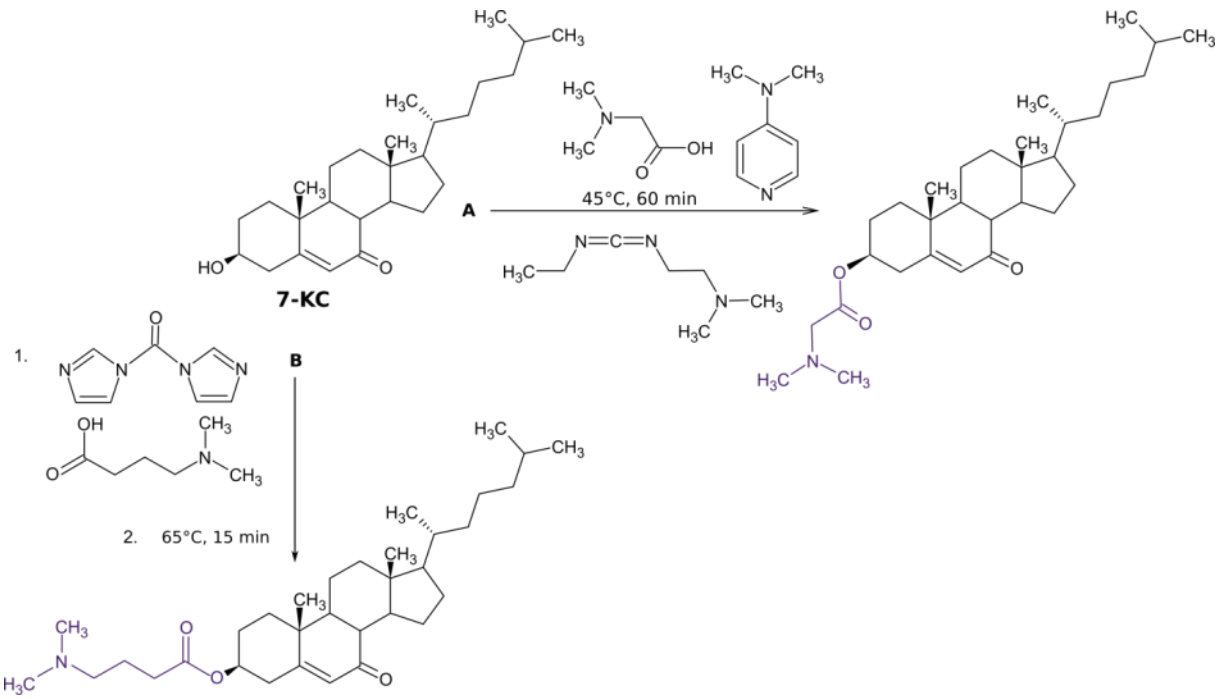

Figure 18| Oxysterol derivatisation reactions (Part 2) A. Derivatisation to N,N-dimethylglycine esters B. Derivatisation to dimethylaminobutyrate esters. Figure adapted from Griffiths *et al.* (2013) [222].

1.1.8.2.1.2.1 DERIVATISATION TO GIRARD HYDRAZONES

Shackelton *et al.* were the first to make use of the very well-known steroid derivatisation with Girard hydrazine reagents introducing in this way a quaternary nitrogen group in the molecules favouring positive ionisation [298–300]. Griffith *et al.* applied the cholesterol oxidase conversion of 3β-hydroxy-5-ene sterols to 3-oxo-4-ene sterols to obtain compounds susceptible for Girard P derivatisation [271,272]. This approach was named as "Enzyme assisted derivatisation for sterol analysis" or also EADSA in literature. For plasma oxysterol analysis, the method consists in the following steps: ethanol protein precipitation accompanied by sonication, first solid phase extraction (SPE), enzymatic conversion of a fraction by the cholesterol oxidase, addition of Girard P reagent reagent to all fractions, second solid phase extraction and finally instrumental analysis. The 17 minutes separation was made on a Hypsersil Gold reversed phase column (1.9 µm particle size, 50 mm x 2.1 mm, Thermo Fisher) with mobile phases A and B being a mixture of methanol and acetonitrile both containing 0,1% formic acid. Oxysterols were detected by a high resolution APCI-linear trap quadrupole (LTQ)-Orbitrap hybrid mass spectrometer using up to five transitions over the course of the chromatographic run and MS/MS/MS also written as MS^3 to even MS^4 scans in some cases. Quantification was made using stable isotope dilution with three IS, one for side-chain oxysterols, one for B-ring oxysterols and the last one for dihydroxycholesterols. The drawbacks of this procedure are the enzyme activity with possible side-reactions, the prerequesit of a free hydroxyl group at position 3 of the sterol ring, the large excess of derivatisation reagent requiring a second solid phase extraction, syn and anti-forms per oxysterol with different retention times, and the laborious nature of the sample preparation protocol [222,224,301]. In the meantime the "Enzyme assisted derivatisation for sterol analysis" was modified by different groups with reduced sample preparation times. The "Enzyme assisted derivatisation for sterol analysis" variants are still called "Enzyme assisted derivatisation for sterol analysis", even if strictly spoken they aren't any more. DeBarber *et al.* for example were interested to analyse three oxysterols in the context of CTX and could omit the enzyme conversion step since it was not necessary in their case. They decided also to bypass the cholesterol removal step, to reduce the amount of Girard P reagent and to cut the derivatisation time to 2h. A trap column in front of the analytical column removed the excess reagent instead of using a second solid phase extraction [302]. Similar to DeBarber *et al.,* Roberg-Larsen *et al*. omitted the cholesterol removal step and used prior to the analytical column, a trap column for removal of excess Girard T, instead of the Girard P reagent [303].

1.1.8.2.1.2.2 DERIVATISATION TO PICOLINYL ESTERS

Honda *et al.* developed a method for derivatisation of hydroxyl groups of oxysterols by picolinyl esters. Only 5 µL of serum were subjected to acid hydrolysis followed by n-hexane extraction. To the dried oxysterol extract a freshly prepared derivatisation mixture of 2-methy-6-nitrobenzoic anhydride, 4-dimethylaminopyridine, picolinic acid, pyridine and triethylamine was given and incubated for 1h at 80°C. Addition of n-hexane allowed the precipitation of excess derivatisation reagents. The precipitated derivatisation chemicals were then removed by centrifugation and the cleaned oxysterol picolinyl esters were dried, redissolved and injected into a LC-ESI-MS/MS system. This derivatisation protocol greatly improved the analytical sensitivity (LOD 5-25 amol on-column for oxysterol dipicolinates and epoxycholesterol picolinate) but oxysterol discrimination still relied on good LC separation [303].

1.1.8.2.1.2.3 DERIVATISATION TO N,N-DIMETHYLGLYCINE ESTERS OR DIMETHYLAMINOBUTYRATE ESTERS

Jiang *et al*. developed an oxysterol derivatisation protocol for the analysis of C-triol and 7-KC for the Niemann-Pick type C (NP-C) diagnostics. 50 µL of plasma were subjected to protein precipitation by methanol, centrifuged, the protein precipitate was removed and the supernatants were dried down. To the dry oxysterol extract dimethylglycine, 4-dimethylaminopyridine, and subsequently 1-ethy-3-(3-dimethylaminopropyl) carbodiimide dissolved in chloroform were added. The mixture was heated for 1h at 45°C, quenched with methanol, dried down and redissolved prior to LC-APCI-MS/MS analysis. The MRM fragments of triol, 7-KC and their respective deuterated standards corresponded to the loss of dimethylglycine $[M+H-103]^+$ and of the protonated dimethylglycine at m/z 104 [285,304,305]. Recently, a similar protocol for oxysterol derivatisation in the context of NP-C diagnosis was reported, with the main difference that dimethylaminobutyrate was used as derivatisation reagent instead of N,N-dimethylglycine [228].

1.1.8.2.2 SELECTIVITY IMPROVEMENT FOR OXYSTEROL ANALYSIS

1.1.8.2.2.1 BENEFICIAL USE OF RELEVANT INTERNAL STANDARDS

Appropriate IS should be used for correct quantification with ideally each target analyte being quantified against its own isotope labelled counterpart [222]. IS fulfil several roles at a time: first they compensate for loss during sample preparation, second they compensate for injection volume variation into the HPLC column, third they help to determine the variation in the retention time of each target analyte among different samples and runs, and finally they help for target analyte identification when peaks are small or not completely separated from interfering peaks [286]. Specific deuterated sterols are considered to be the most ideal IS for HPLC-MS analysis and the structurally closest IS should be used as surrogate if a specific deuterated sterol is commercially not available [225,295]. Alternatively, the surrogate can be a compound not naturally occurring in human serum, as for example coprostanol for cholesterol quantification, since coprostanol is only synthesised by intestinal bacteria and is thus not present in human serum [306].

1.1.8.2.2.2 SAMPLE CLEAN-UP

The biggest issue in oxysterol analysis, especially for non-MRM methods, is that samples usually contain 10^2 to 10^4 times more cholesterol than oxysterols [286]. Separation of cholesterol and oxysterols can be best achieved by SPE allowing a more selective separation of similar structures than for example liquid-liquid extraction. However, complete separation is difficult, especially for some less polar oxysterols. Often mainly nonpolar compounds such as phospholipids responsible for most matrix effects are removed instead of cholesterol [307]. There is not one SPE cartridge that would be the best to use for cholesterol separation, although several groups compared different SPEs stationary phases and brands. Mendiara *et al.*, suggest the use of Zirconia-coated

silica sorbent SPE cartridges from Sigma-Aldrich, while McDonald *et al.* used aminopropyl SPE columns from Biotage [226,307]. Crick *et al.* first advised C18 reversed-phase cartridges (Sep-Pak tC18 200-mg cartridges from Waters) but their inter-batch variation convinced them to change for polymeric hydrophilic-lipophilic balanced reversed-phase cartridges (Waters Oasis HLB cartridges) [308].

Depending on the research question, it might be interesting to unravel the oxysterol esterification degree. For this, the sample is subjected to alkaline hydrolysis and the esterification degree is calculated as the difference between total and free oxysterols. In plasma, 7α,25-OHC and 7α,27-OHC were found to have an endogenous esterification degree of 74 % and 82 %, respectively [220]. Esterification is the mechanism of the cell to inactivate oxysterols. Hence, measuring the total oxysterol content is probably not relevant if the interest is in oxysterol's biological activity.

In summary, if one wants to analyse total oxysterols levels using a derivatisation step to optimize sensitivity and including a cholesterol and phospholipid removal step to minimize matrix effects, the workload per sample is quite high and the procedure difficult to automatise. **Figure 19** summarises the typical work flow of plasma sample preparation for oxysterol analysis.

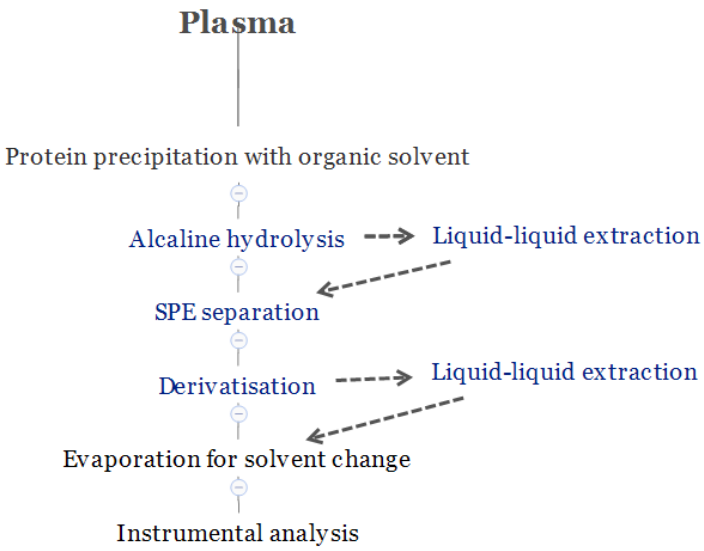

Figure 19| Typical work flow of plasma sample preparation for oxysterol analysis with optional steps highlighted in blue.

1.1.8.2.2.3 LC SEPARATION

Since oxysterols tend to have isobaric mass and are positional isomers, their analysis relies on a good LC separation. Sterols can be separated on both normal and reverse stationary phases but the later is favoured since the reproducibility is higher and the polar mobile phase favours ionisation. Obtaining a good chromatographic oxysterol separation is challenging and co-elution of e.g. 24(S)-OHC and 25-OHC is often occurring [295]. Even with increased choice of performance stationary phases, especially with the arrival of new selective UPLC and monolithic columns, separation of all oxysterols in a single run is still impossible. This separation problem should be considered when looking at quantitative data of oxysterol levels, which might be biased by one or several co-eluting oxysterol(s). For non-MRM methods, it should additionally be assured that cholesterol, still remaining after the poorly selective SPE step is not coeluting with one or several oxysterols [309].

1.1.8.2.2.4 SELECTION BY MS/MS

Today the most selective detection system is the triple quadrupole mass spectrometer, which is also highly sensitive for sterol quantification. Using MS/MS transitions at specific collision energies eliminates interfering analytes. In combination with retention times and the handling of IS, the use of multiple SRM pairs for single sterols adds confidence to the compound identification. The higher the resolution of the mass spectrometer, the better the signal to noise (S/N) ratio and the less interfering peaks appear in the chromatogram. In order to have excellent selectivity, Griffith *et al.* worked with high-resolution MS (HRMS) by a hybrid quadrupole-TOF mass spectrometer or with high-resolution MS^n by a hybrid linear ion-trap/Fourier transform mass spectrometer [292,301]. However, these mass spectrometers do not achieve the same sensitivity and dynamic range for quantification as the triple quadruple mass spectrometer does [309].

1.2 BACKGROUND ON NP-C

1.2.1 INTRODUCTION TO NIEMANN-PICK DISEASES

The first descriptions of one of the diseases known nowadays as Niemann-Pick disease (NPD), date back to 1914, and were written by the German paediatrician A Niemann. He described a 1 ½ year old girl with hepatosplenomegalie and impaired brain and nervous system function. The pathologist L. Pick carried-out postmortem histochemical analysis on the spleen of Niemann's patient. Based on the cholesterol cell load he proved that the patients' disease was different to the lipidosis known at that time [310,311]. Later in 1958, Crocker and Farber published a series of 18 NPD cases and were the first to classify the patients into different subgroups according to clinical and biochemical criteria [312]. Nowadays NPD patients are divided into two diseases with one disease group classified in two categories: Niemann-Pick type C (NP-C), and Niemann-Pick type A (NP-A) and B (NP-B) [313]. All NPD are rare lysosomal storage diseases with neurological implications presenting a large panel of disease unspecific symptoms. The disease course varies between patients but generally patients with milder disease forms may live to their adulthood, while severe disease forms are fatal in toddlerhood. NP-A and B prevalence is estimated to be 1 to 250 000 births and is due to mutations of the acid lysosomal sphingomyelin phosphodiesterase 1 (SMPD1) also known as acid sphingomyelinase (ASM) [314]. This enzyme is involved in the last step of the lysosomal degradation pathway of glycosphingolipids and breaks down sphingomyeline to ceramide and phosphorylcholine. When dysfunctional, sphingomyelin accumulates in the lysosome. NP-A frequently-encountered symptoms are hepatosplenomegaly, neurological deterioration, respiratory infections and a cherry-red spot of the macula of the retina. NP-B is the milder form of NP-A, with later onset and less neurological implications. NP-A and B are diagnosed by ASM enzyme activity testing and are confirmed by molecular genetic testing of the ASM gene [314]. Nowadays both diseases can only be treated symptomatically, but Genzyme (a Sanofi company) started this year a phase 2/3 study for an enzyme-replacement therapy product [315].

1.2.2 GENERAL CONSIDERATIONS ON NIEMANN-PICK TYPE-C

In contrast, NP-C disease is caused by autosomal recessive inheritance of mutations in either the NPC1 or in 5 % of the cases in the NPC2 gene [313,316]. The encoded proteins are responsible in a hand-in-hand manner to transport cholesterol out of the lysosome for further cellular processing [317,318]. If dysfunctional, the intracellular lipid trafficking is impaired and cholesterol and glycosphingolipids accumulate in the lysosomes. The disease is estimated to occur in 1 per 120 000 births and has an extremely heterogeneous clinical presentation [316]. The symptoms comprise systemic and neurological signs being neither age-, nor NP-C -specific and the disease progression occurs at different rates. Examples of symptoms are neonatal cholestasis often associated with spleno- or hepatosplenomegaly, impaired gait, dysarthria, dysphagia, dystonia, gelastic cataplexy, progressive cerebellar ataxia and progressive dementia [313,316]. NP-C is nowadays treated with an iminosugar called Misglustat (N-butyldeoxynojirimycin, Zavesca®) from Actelion Pharmaceuticals, in addition to symptomatic treatment options. The drug was first designed for Gaucher Disease (GD) since it acts as competitive inhibitor of the glucosylceramide synthase, catalysing the first step in glycosphingolipid synthesis. Since glycosphingolipids are also accumulating in the lysosome of NP-C patients, the drug is used for NP-C since 2009 in Europe and proved to stabilise and/or reduce the disease progression rate in NP-C [313,316]. Hydroxypropyl-β cyclodextrin (HPβCD), another drug, is tested in a clinical trial since November 2012 on the initiative of a mother with NP-C affected twins. Direct HPβCD administration into the brain of NP-C presymptomatic and symptomatic cats slowed down disease progression significantly, being therefore a very new promising NP-C drug [319–321]. The mechanism behind HPβCD efficiency in NP-C treatment is poorly understood but appears to be mediated through the activation of the transcription factor EB (TFEB), which is a master regulator of lysosomal biogenesis and autophagy [322]. Laboratory diagnosis of NP-C follows an algorithm proposed by Vanier in 2010 and was revised by Patterson *et al.* in 2012. The general concept is to first undertake useful preliminary tests such as the chitotriosidase assay, or enzymatic testing to exclude Gaucher disease and ASM deficiency, or

assessing the presence of foam cells in the bone marrow. The second step consists in sampling the gDNA and undertaking skin biopsies of the patient for fibroblast culture. Filipin staining of free cholesterol on the cultured fibroblasts should be done in parallel to the genetic testing on gDNA. Finally, the interpretation occurs on all combined results. However, these consecutive tests lack sensitivity and specificity and even mutation analysis is not always conclusive [313,316]. In 2010, Porter *et al.* discovered two autoxidative oxysterols in the plasma of NP-C patients with diagnostic potential [26,285].

1.2.3 RELATIONSHIP BETWEEN NP-C AND OXYSTEROLS

C-triol, and 7-KC found to be specific in NP-C and interestingly correlate with the cholesterol accumulation in the cells of NP-C patients. However, the exact physiological mechanism explaining this correlation in NP-C is still unclear. Three different observations might be a starting point to answer the question of the oxysterol-cholesterol relationship in NP-C disease. The first evidence is that the NPC1 transporter protein has higher binding affinity for oxysterols than cholesterol [323]. Secondly, the oxysterol-binding protein-related protein 5 (ORP5) is necessary for lysosomal trafficking of cholesterol [324]. This last evidence suggests the requirement of oxysterols for cholesterol lysosomal export and the cellular oxysterol synthesis to compensate for the high cholesterol amount. Finally the third evidence and probably in relation to the second point, is the increased cellular oxidative stress due to the cholesterol lysosomal accumulation and due to other cellular dysfunctions observed in NP-C cells leading to ROS formation contributing in this way to non-enzymatic oxysterol formation [325].

1.2.4 *IN VIVO* FUNCTION OF THE NPC PROTEINS

LDL delivers cholesteryl esters to the cell via receptor-mediated endocytosis. Due to endosomal acidification, LDL dissociates from its receptor, which recycles back to the plasma membrane for re-utilisation. The LDL is delivered to the late endosomes/lysosomes where the cholesteryl esters are hydrolysed by the lysosomal acid lipase (LAL) to form free cholesterol [326]. The cholesterol is loaded on a small soluble cholesterol binding protein, called Niemann-Pick C2 protein (NPC2) and subsequently shuttled to Niemann-Pick C1 protein (NPC1) [317,318]. NPC1 is a large glycoprotein with 13 transmembrane domains and one sterol sensing domain and is located in lysosomal membranes [327]. By still badly-known mechanisms, cholesterol is transported to various organelles for further processing. The current knowledge indicates that NPC1 works in association with ORP5 for cholesterol ER targeting [324,328,329]. ORP1L acts as cholesterol sensor for the NPC1-ORBP5 mediated cholesterol transport to the ER membrane. In addition ORP1L recruits proteins essential for placing lysosomes to the perinuclear region into proximity of the ER, enabling for effective cholesterol transfer [330–333]. Interestingly, the cholesterol transport mechanism to the mitochondria seems to involve NPC2 and metastatic lymph node protein 64 (MLN64) but not NPC1, which might explain the observed increase of mitochondrial cholesterol in NPC cells [334,335]. In contrary to the NPC1 or NPC2 mediated cholesterol transport mechanisms, cholesterol moves to the Golgi implicating the trans-Golgi network (TGN)-specific soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), but not the NPC1 or NPC2 proteins. Mutations of either NPC1 or NPC2 as well as other proteins implicated in lysosomal cholesterol sorting, lead to lysosomal cholesterol accumulation and are responsible for the very similar phenotypes observed in other lysosomal storage disorders. Besides the harmful effect of NPC1 mutations in NP-C, it was recently shown that dysfunctional NPC1 completely stops Ebola virus cell entry. How Ebola virus use NPC1 to enter the cell is unclear but is currently under investigation since time-dependent NPC1 auto-inactivation would be an interesting therapeutic options for future Ebola epidemics [330,336,337].

1.3 BACKGROUND ON IBD

1.3.1 GENERAL CONSIDERATIONS ON IBD

Inflammatory bowel diseases (IBD) are a group of chronic relapsing immune-mediated disorders of the gastrointestinal tract that are pathologically characterised by intestinal inflammation and epithelial injury [338,339]. The pathophysiology of IBD will be described in the next section. IBD onset typically occurs in the second or third decade of life and affects an estimated 2,5–3 million people in Europe [340,341]. Frequent symptoms during the active inflammatory state called flare ups are tiredness, weight loss, fever, night sweats, decreased appetite, nausea, vomiting, stomach pain, flush, and (bleeding) bowels. The disease activity is categorised from mild to severe [342,343]. Diagnosis of IBD cannot be made straightforward with only one definitive diagnostic test selective for one particular disease. Instead, the diagnosis consists in the combined assessment of clinical history, and endoscopic, histological or radiological results, as well as laboratory results such as C-reactive protein, erythrocyte sedimentation rate, complete blood count, and fecal calcprotectin measurements [342,344–346]. Using these diagnostic procedures, it is often possible to make a firm diagnosis of IBD. Nevertheless, there are about 10 % of the patients in which the diagnosis isn't certain and which are considered to have indeterminate colitis. The treatment options will depend on the patient's clinical history, the physical findings, and the exact diagnosis. Common treatment strategies include 5-aminosalicylic acid, corticosteroids, immunosuppressive and immunoregulatory agents like azathioprine, anti-TNF therapy or the use of antibiotics and probiotics as well as adequate diet [346]. Crohn's disease (CD) and Ulcerative colitis (UC) are the two most important representatives of IBD, with shared characteristics [347]. UC inflammation is exclusively localised to the colon and mainly starts from the rectum from where it progresses. UC macroscopic mucosa morphology shows perforation of the colon, erythema and friability of the mucosa, superficial ulceration and appearance of pseudopolyps [347]. In CD lesions of the mucosa are discontinuous and might occur anywhere in the gastrointestinal tract but rectal involvement is rare. CD macroscopic mucosa morphology shows segmental transmural inflammatory lesions, linear ulceration, deep into the tissue going inflammation, and appearance of fistulae [347]. IBD etiology remains unknown but appears to be multifactorial implicating a tight interplay between human intestinal microbiome and the mucosal immune system, with an important genetic predisposition. Additionally, the exposome influence appears to be very important, meaning all environmental factors to which a human has been exposed during lifetime such as dietary factors, air and water pollution, food additives, lifestyle factors, stress, hygienic conditions and drug treatment [348].

1.3.2 IBD INITIATION AND PATHOPHYSIOLOGY

The exact mechanism of IBD's initiation is still under investigation and it remains unclear whether changes of the intestinal microbiota composition are the cause or the consequence of impaired mucosal immune function [346,349,350]. Current evidence supports both hypotheses. Family aggregation of IBD was a first hint for genetic susceptibility, and currently 163 IBD risk loci have been identified by genome wide association studies (GWAS). The genetic susceptibility would be an argument for impaired mucosal immune function as starting point of IBD, but a recent publication in *Nature Communication* observed a shift of the microbiota composition in mice towards *Escherichia coli* (*E. coli*) overgrowth suggesting the microbiota shift as possible IBD trigger. The authors discovered that sialic acid availability on intestinal mucosa is, as a nutritive glycan, supporting *E.coli* overgrowth. Higher sialic acid levels on intestinal mucosa would be in turn due to the increased enzymatic sialidase activity of non- destructive bacteria species that proliferate when the mucosa is injured [351]. This study was made by mean of dextran sulphate sodium (DSS)-induced colitis on 2,3 sialyltransferase (St3gal4) (ST) knockout mice as an approach of IBD model. Whatever IBD's causes are, innate immune response is at the origin of the excessive activation of adaptive immunity. However, it is the latter and its associated cytokines that drive deregulation within the epithelial layer [340]. In simple terms, sentinel cell populations, among others the antigen-presenting cells (APC) like dendritic cells (DC) are continuously monitoring the intestinal mucosa for luminal microbes [340]. In normal situation, DC expresses pattern recognition receptors (PPRs) such as toll-like

receptors (TLRs) on their cell surface or nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs) in their cytoplasm [352,353]. TLR4, recognises and is activated by lipopolysaccharides (LPS) from gram-negative bacteria like *E. coli* [354]. The activation results in nuclear translocation and activation of the pro-inflammatory transcription factor Nuclear factor-κβ which in turn will produce pro-inflammatory cytokines and chemokines [355,356]. On the contrary, the cytosolic NOD2 NLR is expressed in intestinal epithelial cells (IECs) and paneth cells, and senses bacterial muramyl dipeptide (MDP). NOD2 activation results, among others in the production of antimicrobial peptides like defensins and hence preserves the balance between the gut commensal communities and plays a protective role during intestinal damages [357,358]. The right balance of the two PRR downstream effects contributes to homeostasis of the intestinal immune system. However, if the balance is impaired favouring TLR4 activation, APCs release pro-inflammatory cytokines such as IL-6, TNF-α, IL-12, and IL-23 which activate T cells of the adaptive immune system [339]. The activated T cells on their turn secrete pro-inflammatory cytokines resulting in the inflammatory signalling cascade. T_H1 cells are known to release IFN-γ, IL-6 and TNF-α, while T_H2 cells are secreting IL-5, IL-6, IL-13 and TNF-α [339]. Interestingly Crohn's disease appears to be predominantly a T_H1 and T_H17-mediated process, while UC seems to be an atypical T_H2 disorder [359].

1.3.3 RELATIONSHIP BETWEEN IBD AND OXYSTEROLS

Several evidences showing a relation between oxysterols and inflammatory disorders such as IBD have appeared throughout the last years. They all concerns different oxysterol receptors, which would suggest oxysterols implication in immunity not to be minor even if actually not much is known. The oxysterol receptors are the transmembrane receptor EBI2, the nuclear receptors LXRα and β and the RORγt.

1.3.3.1 VIA THE TRANSMEMBRANE RECEPTOR EBI2

As mentioned earlier, 7α,25-OHC was found to be the most potent EBI2 ligand on follicular naïve B cells and acts as a B cell chemoattractant in follicular microenvironments [28,35]. Additionally this EBI2-7α,25-OHC system plays a role in the positioning of splenic cluster of differentiation (CD) $CD4^+$ DC in the marginal zone of the bridging channels to prime T cells for an efficient antibody response [360,361]. Recently, in addition to B cells and DC, macrophages were also demonstrated to migrate in an EBI2-depenent manner [362]. EBI2 mRNA was furthermore found in T cells, natural killer cells (NK-cells), neutrophils, basophils and eosinophils [28]. However, in these cells, no 7α,25-OHC promoted-motion could be shown, indicating different inflammatory response mechanisms of 7α,25-OHC. At the moment it appears that the chemotactic feature of EBI2 is due to the combined action of two EBI2 signalling pathways: the Gαi dependent signalling pathway and the β-arrestin recruitment pathway being G protein-independent. However, the Gαi dependent signalling pathway appears to have a more prominent action than the β-arrestin recruitment pathway [363]. Other EBI2 downstream effects have been reported supporting the hypothesis that 7α,25-OHC triggers several inflammatory responses. Among the different downstream signalling pathways are calcium release, guanosine 5'-O-[gamma-thio]triphosphate (GTPγS) binding, p38 mitogen-activated protein kinase activation and extracellular signal-regulated kinase 1/2 (ERK1/2) mitogen-activated protein kinase activation [28,35,128,129,363]. The occurrence of several downstream pathways for the EBI2-7α,25-OHC system indicates that it is an important immune regulator which needs to be tightly controlled. Besides 7α,25-OHC, 7α,27-OHC and 7β,25-OHC were also shown to bind to the EBI2 receptor but not as strong as 7α,25-OHC does.

Three different observations implicate the EBI2 – dihydroxycholesterol system to have a role in the IBD human pathophysiology. The first and most important is the identification by Jostins *et al.* in 2012 that EBI2 is an IBD risk gene and that the T allele of the single nucleotide polymorphism (SNP) rs9557195 increases the risk for both, CD and UC with genome wide significance [364]. Secondly, a single nucleotide polymorphisms (rs9585056) in the EBI2 gene was found to occur in inflammatory diseases such as type 1 diabetes [365]. Finally increased EBI2 receptor levels were shown in the ileum of CD patients with NOD2 mutations [366]. Taken together these data strongly suggests not only an implication but although a protective role for the EBI2 receptor in IBD. EBI2 is at

the current state of knowledge activated by dihydroxycholesterols, and therefore these oxysterols are of high interest for the IBD research.

1.3.3.2 VIA NUCLEAR RECEPTORS

1.3.3.2.1 VIA LIVER X RECEPTORS

The nuclear receptors LXRs are as already mentioned established regulators of lipid-inducible gene expression and are activated by a certain number of oxysterols, among them 24-OHC, 25-OHC and 27-OHC. However, the discovery of LXRs implication in immunology is just in its beginning and started in 2003 with two distinct studies. Both studies could show that the synthetic LXR agonist GW3965 inhibited the *in vitro* as well as the *in vivo* macrophage response to bacterial infections or to lipopolysaccharide (LPS) stimulation. The synthetic LXR agonist GW3965 inhibited macrophage expression of pro-inflammatory mediators, such as IL-1β, IL-6, metalloproteinase 9, nitric oxide synthase (iNOS), cyclooxygenase 2 (COX-2), monocyte chemoattractant protein-1 (MCP-1), MCP-3, macrophage inflammatory protein 1β (MIP-1β) and interferon inducible protein-10 (IP-10) [367,368]. Moreover, inflammation in murine models of contact dermatitis and atherosclerosis was reduced by synthetic LXR ligands [367,369,370]. Only recently Jakobssen *et al.* showed an interconnection between LXR activation and inhibition of pro-inflammatory cytokines in IBD, with a more noticeable protective role for LXRβ than LXRα. They found that the expression of both LXRα and LXRβ were significantly suppressed in inflamed colons from IBD subjects compared to non-inflamed colons. The receptors were thus not able to exert their protective action in IBD patients. Based on their findings, the authors suggest LXRs to be an interesting pharmaceutical target to reduce the inflammatory state in IBD [371].

1.3.3.2.2 VIA RAR-RELATED ORPHAN RECEPTOR GAMMA T

A second nuclear receptor, RORγt was proven to be activated by oxysterols with the most potent being 7β,27-OHC, which accelerated differentiation of pro-inflammatory T helper 17 (T_H17) cells [135]. T_H17 cells are implicated in various chronic inflammatory diseases among others, CD and UC [372–376]. At the moment, there are very contrasting findings on the role of T_H17 cells in UC. There are suggestions for pathological as well as protective roles [377–381]. This T_H17 function discrepancy might be explained by the fact that the T_H17 cells pathogenic capabilities depend on the environment of IL-17 production. If activated in presence of IL-23, T_H17 cells are more aggressive than T_H17 cells activated with IL-6 and Transforming growth factor-β1 (TGF-β) [382,383]. A minority of IL-6 and TGF-β-activated T_H17 cells even expressed the anti-inflammatory IL-10, thus contributing to the discovered protective action of T_H17 cells. [384].

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2. RESULTS

2.1 LC-MS/MS BASED ASSAY AND REFERENCE INTERVALS IN CHILDREN AND ADOLESCENTS FOR OXYSTEROLS ELEVATED IN NIEMANN-PICK DISEASES

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2.1.1 ABSTRACT

2.1.1.1 BACKGROUND

Niemann-Pick type C (NP-C) is a rare progressive neurodegenerative lipid storage disorder with heterogeneous clinical presentation and challenging diagnostic procedures. Recently oxysterols have been reported to be specific biomarkers for NP-C but knowledge on the intra-individual variation and on reference intervals in children and adolescents are lacking.

2.1.1.2 METHODS

We established a LC-MS/MS assay to measure Cholestane-3β,5α,6β-triol (C-triol) and 7-ketocholesterol (7-KC) following Steglich esterification. To assess reference intervals and intra-individual variation we determined oxysterols in 148 children and adolescents from 0-18 years and repeat measurements in 19 of them.

2.1.1.3 RESULTS

The reported method is linear (r >0.99), sensitive (detection limit of 0.03 ng/mL [0.07 nM] for C-triol, and 0.54 ng/mL [1.35 nM] for 7-KC) and precise, with an intra-day imprecision of 4.8 % and 4.1 %, and an inter-day imprecision of 7.0 % and 11.0 % for C-triol (28 ng/ml, 67 nM) and 7-KC (32 ng/ml, 80 nM), respectively. Recoveries for 7-KC and C-triol range between 93 % and 107 %.

The upper reference limit obtained for C-triol is 40.4 ng/mL (95% CI: 26.4-61.7 ng/mL, 96.0 nM, 95% CI: 62.8-146.7 nM) and 75.0 ng/mL for 7-KC (95% CI:55.5-102.5 ng/mL, 187.2 nM, 95% CI:138.53-255.8 nM), with no age or gender dependency. Both oxysterols have a broad intra-individual variation of 46 % ± 23 % for C-triol and 52 % ± 29 % for 7-KC. Nevertheless, all Niemann-Pick patients showed increased C-triol levels including Niemann Pick type A and B patients.

2.1.1.4 CONCLUSIONS

The LC-MS/MS assay is a robust assay to quantify C-triol and 7-KC in plasma with well documented reference intervals in children and adolescents to screen for NP-C in the peadiatric population. In addition our results suggest that especially the C-triol is a biomarker for all three Niemann-Pick diseases.

2.1.2 INTRODUCTION

Niemann-Pick type C (NP-C) disease is a neurodegenerative lipid storage disorder caused by dysfunction in one of the lysosomal proteins: NPC1 or NPC2 [1,2]. These proteins are responsible for displacing free cholesterol from the lysosome to other cellular compartments [3–6]. Dysfunction resulting from mutations in either the NPC1 or the NPC2 gene leads to cholesterol accumulation in the lysosome, thereby damaging several organs. Typical symptoms include neonatal cholestasis [7,8] often associated with spleno- or hepatosplenomegaly, progressive cerebellar ataxia, speaking and swallowing difficulty, progressive dementia, and the affected children often die very young [1]. The current diagnostic method is a set of consecutive tests including chitotriosidase assay, filipin staining of free cholesterol in fibroblasts, or genetic testing [1]. Chitotriosidase assay [9–12] and Filipin staining [13–16] lack sensitivity and specificity, and are only performed in a few medical centres [17]. Mutation analysis is a useful diagnostic tool, however, it is time consuming and not always conclusive [18–20].

New hope for a more straight forward diagnosis of NP-C arose from the discovery of higher amounts of Cholestane-3β,5α,6β-triol (C-triol) and 7-ketocholesterol (7-KC) in plasma of NP-C patients than in plasma from patients with other lysosomal storage diseases or from controls [21,22]. These authors showed in addition that plasma concentrations of C-triol and 7-KC directly correlated with the disease state and that these oxysterols are specific biomarkers for NP-C [21]. In particular, the oxysterols were not elevated in several other lysosomal storage diseases like infantile neuronal ceroid lipofuscinosis (INCL), GM-1 gangliosidosis (GM-1), GM-2 gangliosidosis (GM-2), and Gaucher disease (GD). However, a later study indicated that Niemann-Pick type A (NP-A) and B patients (NP-B) may also show increased levels of oxysterols [23]. NP-A and NP-B are sphingolipidoses with a similar clinical picture to NP-C but a distinct pathophysiology demanding specific treatment [16,17,24].

It is not clear why these oxysterols are increased in NP-C. It was recently suggested that the dysfunction of several cellular organelles in NP-C patients would lead to oxidative stress which in turn would increase oxysterol levels [25]. And it was shown that the NPC-1 protein is not only able to bind cholesterol but also some oxysterols [26], indicating a role in oxysterol metabolism.

Although a GC-MS [21] and a LC-MS/MS [22] assay have been developed to measure C-triol and 7-KC in NP-C, there are no data on the intra-individual variability and on reference intervals in children and adolescents for these analytes. Here we describe a robust and accurate method to measure C-triol and 7-KC in plasma. Using this method we determined reference intervals and the intra-individual variability in a large group of children and adolescents, and we show evidence that especially the C-triol is elevated in all three Niemann-Pick diseases.

2.1.3 MATERIAL AND METHODS

2.1.3.1 CHEMICALS

4-(Dimethylamino)pyridine (DMAP), N,N-Dimethylglycine (DMG), 7-Ketocholesterol with > 90 % purity (7-KC), Cholestane-3β,5α,6β-triol (C-triol) with > 90 % purity (estimated by thin layer chromatography), n-Hexane, Ammonium hydroxide solution and Chloroform stabilised with amylenes with Sure/Seal™ system were obtained from Sigma-Aldrich Chemie GmbH, Buchs, Switzerland. 1-Ethyl-3-(3'dimethl-aminopropyl) carbodiimide (EDC) was obtained from VWR International GmbH Dietikon, Switzerland. 25,26,26,26,27,27,27-[$^2H_7$]7-Ketocholesterol (d^7-7-KC) with > 98 % purity was purchased from Santa Cruz Biotechnology, Santa Cruz, CA., USA. 25,26,26,26,27,27,27-[$^2H_7$]cholestane-3β,5α,6β – triol (d^7-C-triol) with > 99 % purity was bought from Avanti® Polar Lipids, Inc. Alabaster (Ala), USA. Methanol absolute was obtained from Chemie Brunschwig AG, Basel, Switzerland.

2.1.3.2 PREPARATION OF INTERNAL STANDARD, CALIBRATORS AND QUALITY CONTROLS

Deuterated C-triol and 7-KC were dissolved in methanol (MeOH) at a concentration of 40 ng/mL and stored at -80°C until use. A stock solution of C-triol and 7-KC in MeOH was prepared at a concentration of 1000 ng/mL. The final concentrations of the calibration points for both oxysterols were 10, 30, 65, 100 and 300 ng/mL.

2.1.3.3 SAMPLE COLLECTION

Anonymised residual heparin samples [27] from children age 0 to 18 years were collected from routine analysis at the University Children's Hospital Zurich. Plasma Samples were immediately frozen and stored at -80°C for a maximum of 4 months before analysis. Patient samples were obtained also in anonymised form from the metabolic units of the University Hospitals with indication of the genetically diagnosed disease only. All metabolic units have approval from their local ethics committees and written informed consent from the patients.

2.1.3.4 SAMPLE PREPARATION

Samples were prepared according to the protocol of Jiang *et al*. [22] with modifications. In brief, 50 µL of plasma were added to 250 µL of internal standard (IS) solution (40 ng/mL in MeOH) and centrifuged for 10 min at 11'300 g. The supernatant was transferred into a new tube and then removed under a nitrogen stream till complete dryness of the probe. To assure reproducible derivatisation, 100 µL of DMAP/DMG (4 M: 1 M) and 100 µL of EDC (1 M) solution were added to form dimethlylglycine esters from alcohols [28,29], the tube capped, vortexed and subjected to 1 min ultrasound before heating in a waterbath at 45°C for 1 h. Successful derivatisation was assessed by the shift of the solution colour from colourless to yellow. The reaction was quenched with 100 µL of MeOH. A liquid-liquid extraction with 2 mL of hexane and 1 mL of 0.1 N ammonium hydroxide was done twice. The samples were dried under a nitrogen stream and reconstituted with 200 µL of MeOH/Water (60:40).

2.1.3.5 LC-MS/MS ANALYSIS

The analysis was performed on an AB Sciex 4000 Q TRAP (AB Sciex, Zug, Switzerland) coupled with a Dionex Ultimate 3000 (Thermo Scientific, Olten, Switzerland), using a Gemini-NX™ C18 column (100 mm x 200 mm; 3 µm particle size and 110 Å pore size from Phenomenex, Brechbühler AG, Schlieren, Switzerland). The following gradient was applied using solvent A (2 mM ammonium formate; pH 2.45) and B (2 mM ammonium formate in MeOH): Solvent B increased from 69 % to 75 % within 3 min, and subsequently increased to 99 % within 4.9

min. After 2 min at 99%, B decreased to 1 % within 1 min and stayed at 1 % for 5 min. B increased from 1 % to 69 % within 30 sec and stayed at 69 % for 1.6 min. The flow rate was 250 µL/min and the injection volume 10 µL.

The source parameters of the AB Sciex 4000 Q TRAP were CAD: Medium, CUR: 15, GS1: 60; GS2: 70, IS: 4500, TEM: 500. Multiple reaction monitoring (MRM) in positive ion mode was used to identify the oxysterols with the precursor and selected product ions listed in **Table 1**. For quantification the first mentioned fragment ion of **Table 1** was used and all ions were monitored with a dwell time of 50 msec.

Table 1: MRM parameters and retention times for C-triol and 7-KC

Positive ion mode $[M+H]^+$ with ESI	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	DP (V)	EP (V)	CE (V)	CXP (V)
Cholestane-3β,5α,6β-triol	4.76 ± 0.05	591.6	488.6	130	10	28	10
			104.1	130	10	45	6
D^7- Cholestane-3β,5α,6β-triol	4.76 ± 0.05	598.6	495.6	130	10	28	10
			104.1	130	10	45	6
7-Ketocholesterol	7.91 ± 0.11	486.6	383.5	110	10	26	10
			104.1	110	10	37	6
D^7-7-Ketocholesterol	7.91 ± 0.11	493.6	390.5	110	10	26	10
			104.1	110	10	37	6

2.1.3.6 LINEARITY, PRECISION AND RECOVERY

The linearity was investigated by a 5 point calibration curve made in plasma. Intra-day (n=6) and inter-day imprecision (n=6) were assessed measuring a low quality control (LQC, human plasma), a medium quality control (MQC: plasma spiked with 30 ng/mL oxysterol) and a high quality control sample (HQC: plasma spiked with 100 ng/mL oxysterol). The absolute recovery of the oxysterols was calculated from the results of the spiked MQC and HQC samples. For calculation of the recoveries endogenous levels of oxysterols (LQC) were subtracted from the MQC and HQC samples. The mean of the recoveries was divided by the spiked amount and multiplied by 100.

2.1.3.7 LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated as follow [30] :

$$LOD = y_{LQC} + 3.3 * s_{LQC}$$

$$LOQ = y_{LQC} + 10 * s_{LQC}$$

With y_{LQC}= y-intercept of the calibration equation

S_{LQC}= standard deviation of the peak area/IS area ratio of the inter-day LQC samples

2.1.3.8 SAMPLE STABILITY

To assess the stability of the C-triol and the 7-KC in plasma, MQC triplicates were stored at 22 °C (middle European temperature) and at 37°C (Southern temperature) and collected at different time points (day 1, day 2, day 3, day 4 and day 5). After collection the samples were frozen at –80°C. The values determined for day 0 were set to 100 %

2.1.3.9 DATA ANALYSIS

Data analysis was made in Microsoft Excel 2010. Correction for endogenous oxysterol levels in the plasma pool used for calibration was made by ignoring the y-intercept of the linear function. To establish reference intervals and age dependency MedCalc® software was used. Logarithmic transformation was required for data normalisation prior to determination of outliers, reference intervals and the investigation of age and gender dependency. Outliers were calculated with the generalised extreme studentised deviate test [31]. Two outliers for C-triol were detected (106.17 ng/mL [252.38 nM] and 286.42 ng/mL [680.87 nM]) and removed from the dataset. Reference intervals were estimated using the double sided non-parametric percentile method (2.5%-97.5%). Since one side of the reference interval was lower than the measuring interval, only the upper limit of the reference intervals is communicated. Age dependency was initially visually evaluated **(Figure 3)** and then calculated using the correlation analysis of MedCalc® with the following age groups: 0-3 months (11 samples), 3-6 months (9 samples), 6-12 months (no samples), 12 months-24 months (5 samples), 24-36 months (7 samples), 36-48 months (12 samples), 4- 6 years (16 samples), 7-12 years (37 samples) and 13-18 years (51 samples). Gender dependency was assessed graphically and using a two-sided t-test in Excel 2010. The intra-individual variation (CV_w) was calculated as the coefficient of variation for each non-NP-C control with repeat measurements. The average coefficient of variation and the standard deviation from all 19 controls with repeat measurements is communicated. For reference intervals values calculation only the average oxysterol concentration for each patient was included. For all statistical tests a p-value < 0.05 was considered significant.

2.1.4 RESULTS

2.1.4.1 METHOD VALIDATION

The quantification of C-triol and 7-KC in human plasma was performed by MRM in positive ion mode using specific transitions for each analyte **(Table 1).** The calibration was linear from 10 to 300 ng/mL (i.e. the observed oxysterol concentrations in patients and controls) with a correlation coefficient > 0.99 for both oxysterols. The assay has a limit of detection of 0.03 ng/mL [0.07 nM] and of 0.54 ng/mL [1.35 nM], while the limit of quantification is 0.08 ng/ml [0.19 nM] and 0.80 ng/ml [2.00 nM] for the C-triol and 7-KC, respectively **(Table 2).**

Table 2: Linearity, limit of detection, limit of quantification, intra- and interday precision, and recoveries for C-triol and 7-KC

	Linear equation	R^2	LOD (ng/mL)	LOQ (ng/mL)	Measuring interval		Intra-day precision (n=6)		Inter-day precision (n=6)	
						QC (ng/mL) [nM]	CV (%)	Recovery (%)	CV (%)	Recovery (%)
Cholestane-3β,5α,6β-triol	y=0.11x+0.02	0.99	0.03	0.08	10-300	LQC (6) [14]	7.8	-	12.4	-
						MQC (28) [67]	4.8	93	7.0	97
						HQC (100) [238]	2.8	100	1.8	102
7-Keto-cholesterol	y=0.35x+0.22	0.99	0.54	0.80	10-300	LQC (16) [40]	14.9	-	24.9	-
						MQC (32) [80]	4.1	107	11.0	103
						HQC (123) [308]	2.6	123	9.1	111

Using this method, the two oxysterols were quantified within 20 minutes with elution times of 4.8 ± 0.05 min for the C-triol (d^7-C-triol and C-triol) and of 7.9 ± 0.11 min for 7-KC (d^7-7-KC and 7-KC) **(Figure 1)**. The specificity was ensured by recording two transitions for each analyte **(Table 1).**

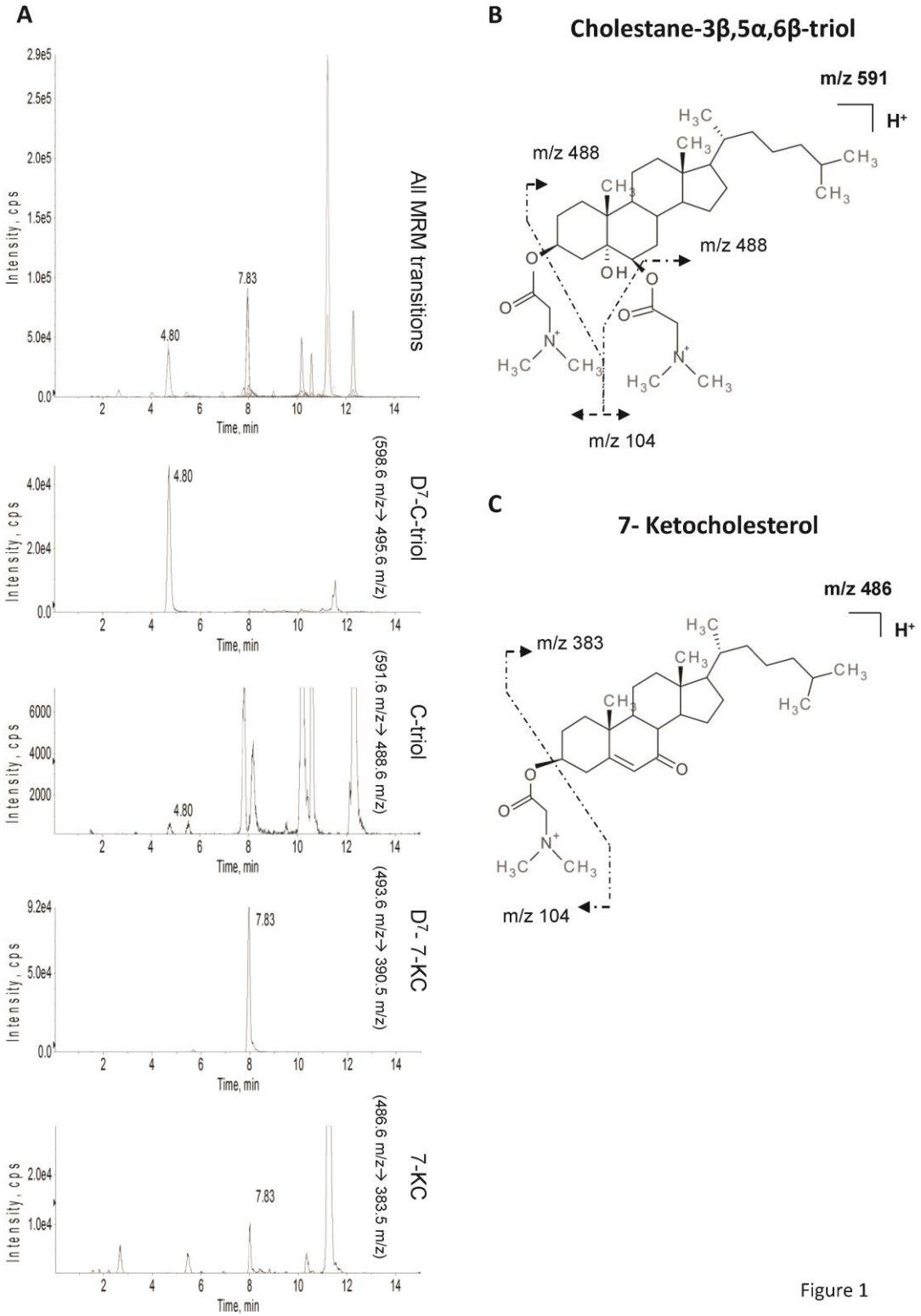

Figure 1

Figure 1. A. MRM transitions chromatograms of Cholestane-3β,5α,6β-triol (retention time of 4.80 min) and 7-Ketocholesterol (retention time of 7,83 min) and their respective deuterated standards in plasma of non NP-C subjects. B. Structure of the derivatised Cholestane-3β,5α,6β-triol molecule with its fragmentation sites following MRM. C. Structure of the derivatised 7- Ketocholesterol molecule with its fragmentation sites following MRM.

The precision of the method was investigated by the intra-day and inter-day imprecision. The imprecision is generally low for both oxysterols with intra-day coefficients of variation (CV) ranging from 2.8-7.8 % for C-triol

and 2.6-14.9 % for 7-KC and with inter-day CVs of 1.8-12.4 % for C-triol and 9.1-24.9 % for 7-KC **(Table 2).** The larger imprecisions were only observed for concentration at the lower limit of quantification.

The accuracy of the method was investigated by analysing recoveries of oxysterols in spiked plasma samples. The recoveries for both oxysterols varied between 93-107 %, excepted for 7-KC in the highest spiked concentration sample, where a recovery of 123 % was observed **(Table 2).**

2.1.4.2 SAMPLE STABILITY

Sample stability assays at 22°C and 37°C were performed to investigate whether plasma samples can be shipped at ambient temperature or will need shipment by overnight mail on dry ice. **Figure 2A**. shows that the C-triol concentration in human plasma is stable for four days at 22°C before decreasing by more than 15 %. In contrast, C-triol is not stable at 37°C with decreased levels detected already after one day. Similarly, 7-KC **(Figure 2B)** is stable in samples stored at 22°C but also decreased already after one day at 37°C. Hence, shipping plasma samples at ambient temperature is only suitable in a moderate climate ensuring delivery within four days.

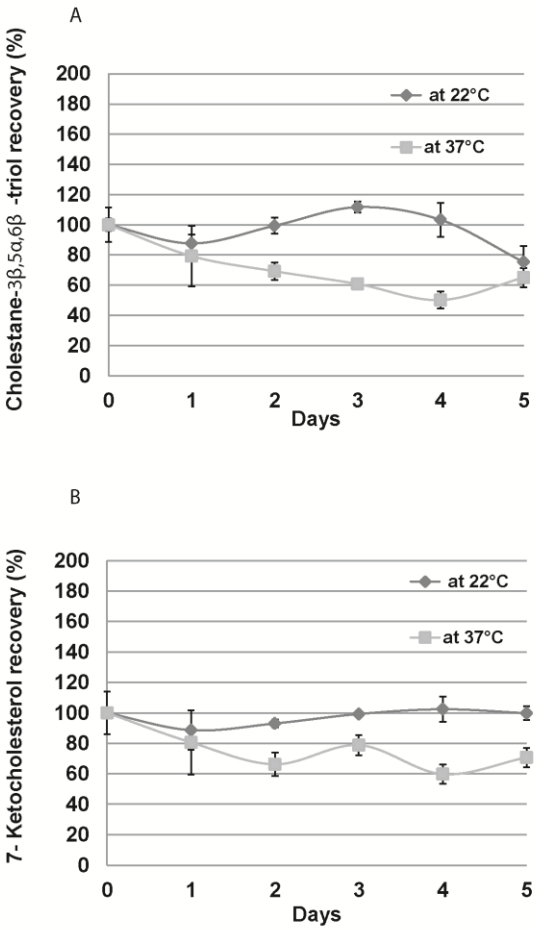

Figure 2

Figure 2. Stability of Cholestane-3β,5α,6β-triol (A) and 7-Ketocholesterol (B) in plasma following storage for 0 to 5 days at 22 °C and 37 °C. The stability of the oxysterols is expressed as percentage of day 0. The error bars indicate the standard deviation of triplicates.

2.1.4.3 REFERENCE INTERVALS IN CHILDREN AND ADOLESCENTS

The reference intervals for the oxysterols in children and adolescents were investigated by analysing plasma samples from 148 non NP-C children ranging from a few days of age to 18 years of age. We determined an upper reference limit of 40.4 ng/mL (95 % CI:26.4-61.7 ng/mL, 96.0 nM, 95 % CI:62.8-146.7 nM) for the C-triol and of 75.0 ng/mL (95 % CI:55.5-102.5 ng/mL, 187.2 nM, 95 % CI:138.53-255.8 nM) for 7-KC with no significant age or gender dependency **(Figure 3)**.

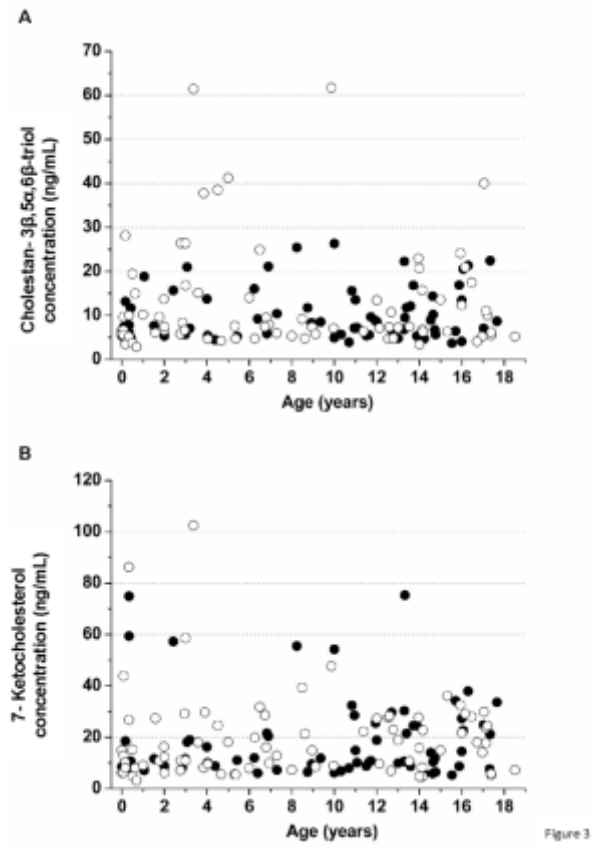

Figure 3. Cholestane-3β,5α,6β-triol (A) and 7-Ketocholesterol (B) concentrations by age and gender of non NP-C children and adolescents. White dots (○) represent boys, black dots (●) represent girls. Conversion factor for Cholestane-3β,5α,6β-triol: 1 ng/mL equals 2.38 nM, while for 7-Ketocholesterol 1 ng/mL equals 2.50 nM

2.1.4.4 BIOLOGICAL VARIATION IN CHILDREN AND ADOLESCENTS

The biological variation of oxysterols in 19 non NP-C children plasma was investigated by measuring different samples of the same volunteer. The number of samples per volunteer ranged between 2 and 5 and the samples were collected within maximally 2 months. A high biological variation was observed for both oxysterols in plasma from non NP-C children with an average CV_w of 46 % ± 23 % standard deviation for C-triol and 52 % ± 29 % standard deviation for 7-KC.

2.1.4.5 OXYSTEROL CONCENTRATIONS IN CHILDREN AND ADOLESCENTS WITH NIEMANN-PICK DISEASES

The diagnostic potential of the oxysterol measurement for NP-C, NP-B and NP-A was investigated, by measuring oxysterol levels in plasma of such patients **(Figure 4)**. The C-triol concentration in plasma of NP-C patients ranged from 108.1-140.3 ng/mL [257.0-333.5 nM], while the concentrations for 7-KC ranged from 113.2 -174.4 ng/mL [282.6-435.3 nM], values which are well above the upper reference limit for both

oxysterols. Similarly, NP-A and NP-B patients showed increased levels for both oxysterols with levels for NP-B ranging from 71.3-631.3 ng/mL [169.5-1500.7 nM] and 75.1-645.7 ng/mL [187.5-1611.7 nM] for C-triol and 7-KC, respectively.

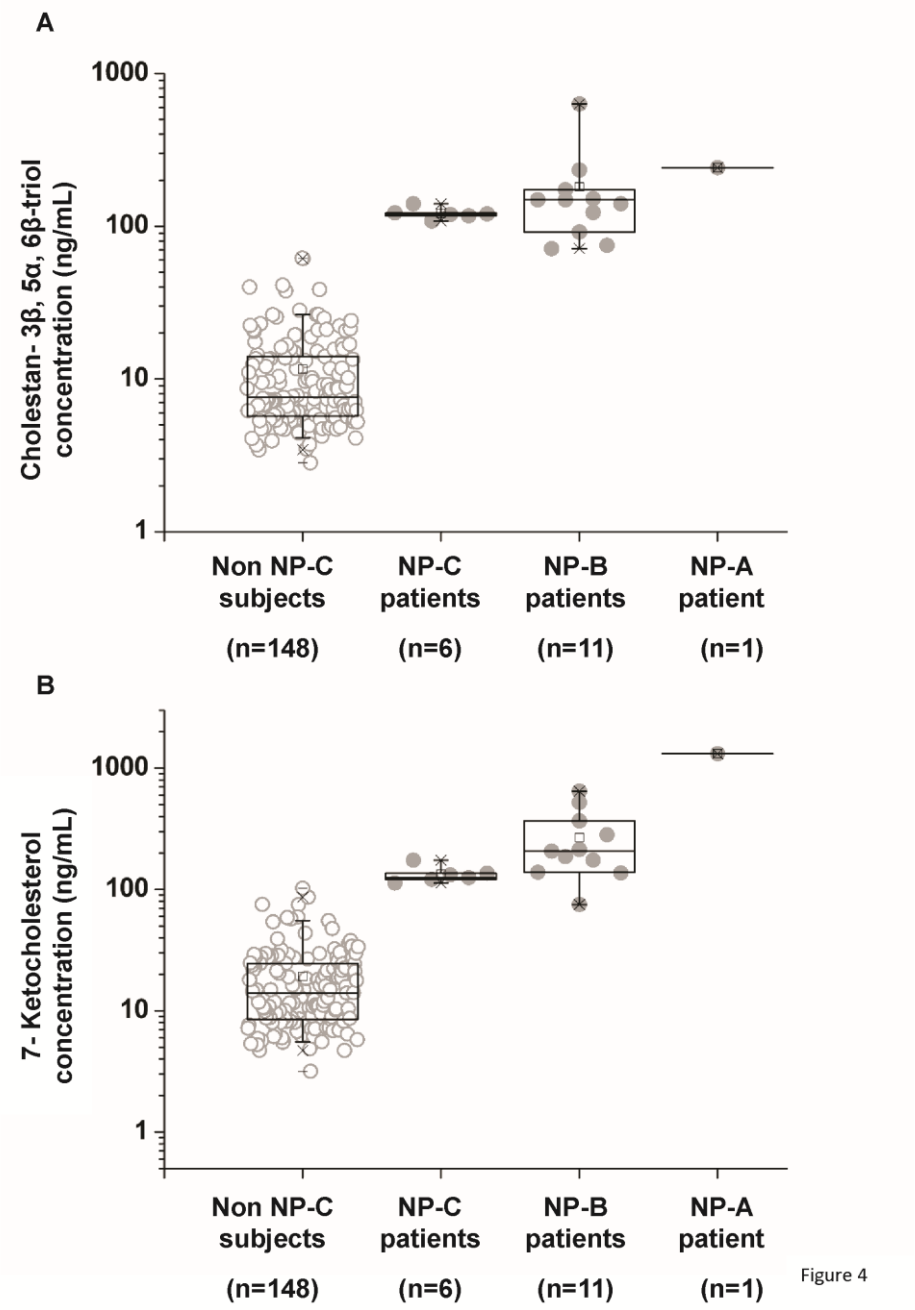

Figure 4

Figure 4. Logarithmic box-and-whisker plot and raw data representation of Cholestane- 3β,5α,6β-triol (A) and 7-Ketocholesterol (B) concentrations in non NP-C children and children with Niemann Pick type C, B and A. Box 25th to 75th percentile, line: median, square: mean, box whiskers: 5th and 95th percentiles, cross: 1th and 99th percentiles, outer ticks: data ranges. Conversion factor for Cholestane-3β,5α,6β-triol: 1 ng/mL equals 2.38 nM, while for 7-Ketocholesterol 1 ng/mL equals 2.50 nM.

2.1.5 DISCUSSION

Here we present a reproducible and robust LC-MS/MS method to measure the oxysterols C-triol and 7-KC in human plasma to screen for Niemann-Pick disease. The assay validation showed good recovery rates (93-123 %) and good precision with CVs of < 15 % for both oxysterols at relevant concentrations. In addition, the method is linear over the entire measuring range, which covers oxysterol levels in plasma of non NP-C and NP-C children and adolescents.

In comparison to previously published methods [22,32], the robustness of our method is secured by an improved sample preparation protocol, in which we ensure the reliability of the derivatisation reaction and decrease the matrix interference by extracting the derivatised oxysterol before analysis. The increase in the concentration of the derivatisation reagent assures a reproducible derivatisation of the sterically hindered C-triol molecule in presence of large amount of functional hydroxyl groups present in plasma. A lower concentration of the derivatisation reagent resulted in variable recoveries for the C-triol due to a presumably non-reproducible derivatisation in the different plasma samples.

Using this method we report the upper reference limit in plasma from non-NP-C children and adolescents. For the calculation of the reference intervals we included oxysterol measurements from non-healthy children and adolescents because we have access to these samples and because such reference intervals better reflect the real diagnostic context in the hospital setting than a selection of healthy volunteers. As a consequence our upper limit of the reference intervals are slightly higher than previously published reference intervals using LC-MS/MS (C-triol: 7.4-21.2 ng/mL [17.6-50.4 nM] and 7-KC: 11.4-44.4 ng/mL [28.5-110.8 nM] [22] and C-triol: 0.8-8.1 ng/mL [1.9-19.3 nM] and 7-KC: 2.6-30.5 ng/mL [6.5-76.1 nM] [33]). Although using such an approach may include one or two not diagnosed NP-C patients in the process of establishing reference intervals, outlier correction and the use of the 97.5 percentiles of the measurements to establish the upper reference limit would omit the influence of such potentially confounding samples. Indeed, all NP-C patients showed elevated oxysterol levels suggesting that the higher reference limits did not reduce the sensitivity of the oxysterol assay to identify NP-C patients. Such a clear separation by the oxysterols of NP-C from non-NP-C patients observed in this sample, however, may overestimate the diagnostic efficiency of the assay. Previous studies have indicated that there are some NP-C patients with C-triol and 7-KC levels in the reference intervals [22].

In addition to the usefulness of the plasma oxysterols to diagnose NP-C disease, we show evidence that the oxysterol levels are also elevated in NP-A and NP-B patients. These findings are in agreement with a recently published study which already reported elevated 7-KC levels in NP-A/B patients [23], indicating that 7-KC is not a specific marker for NP-C but a biomarker for Niemann-Pick patients in general. Our study expands these findings showing that the C-triol plasma levels are also elevated in NP-A and NP-B patients and that the C-triol plasma levels are even more discriminatory to identify Niemann-Pick patients than the 7-KC levels. While some of the NP-B patients showed 7-KC levels under the upper reference limit, none of the Niemann-Pick patients had C-triol levels under the upper reference limit.

Overnight shipment of plasma samples to the metabolic laboratory is often necessary and the stability of the analytes at ambient temperature will determine the pre-analytical precautions necessary for shipment. Our results indicate that samples can be shipped at room temperature by express mail or courier when there are mild temperatures because both oxysterols are stable at 22°C for four days. However, shipment in warmer regions should be done using cooling devices or frozen on dry ice.

In summary, we present a robust LC-MS/MS method to measure two oxysterols in human plasma and we established upper reference limits in children and adolescents, which discriminate between controls and Niemann-Pick patients in general. In addition our results indicate that the C-triol is more discriminative than the 7-KC for the identification of Niemann-Pick patients. The presented method is rapid, non-invasive, quantitative, and specific, facilitating the diagnostic procedures in patients suspected for Niemann-Pick disease.

Particularly, it enables the investigation of children, adolescents and adults in large numbers with neurological disorders, hepatomegaly or splenomegaly for NP disease, improving the diagnostic procedures and facilitating genetic confirmation.

2.1.6 REFERENCES

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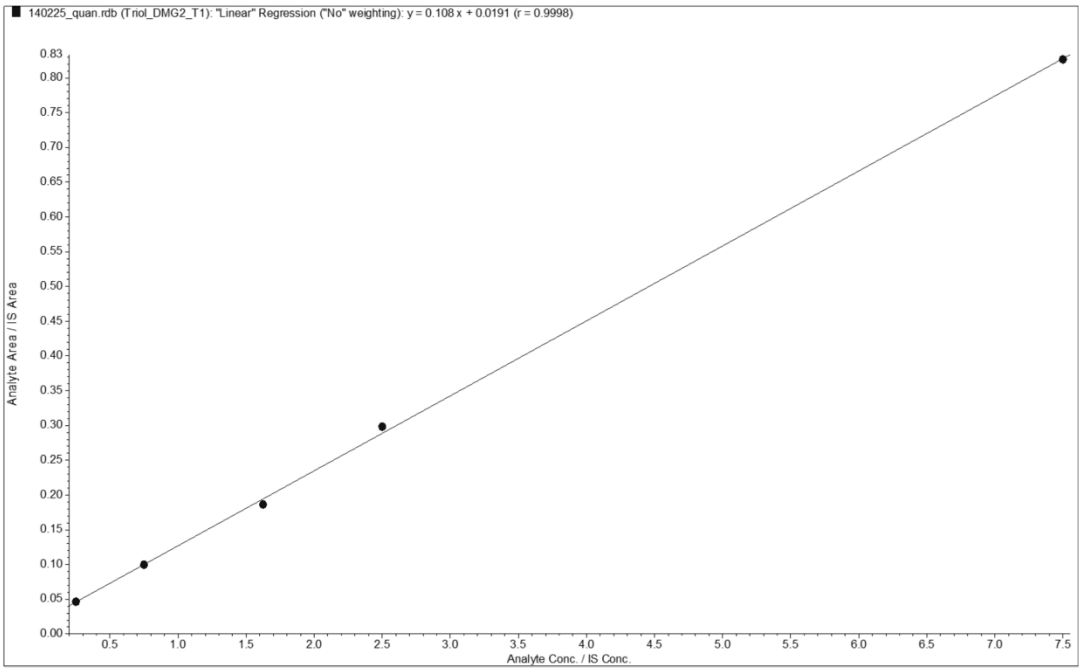
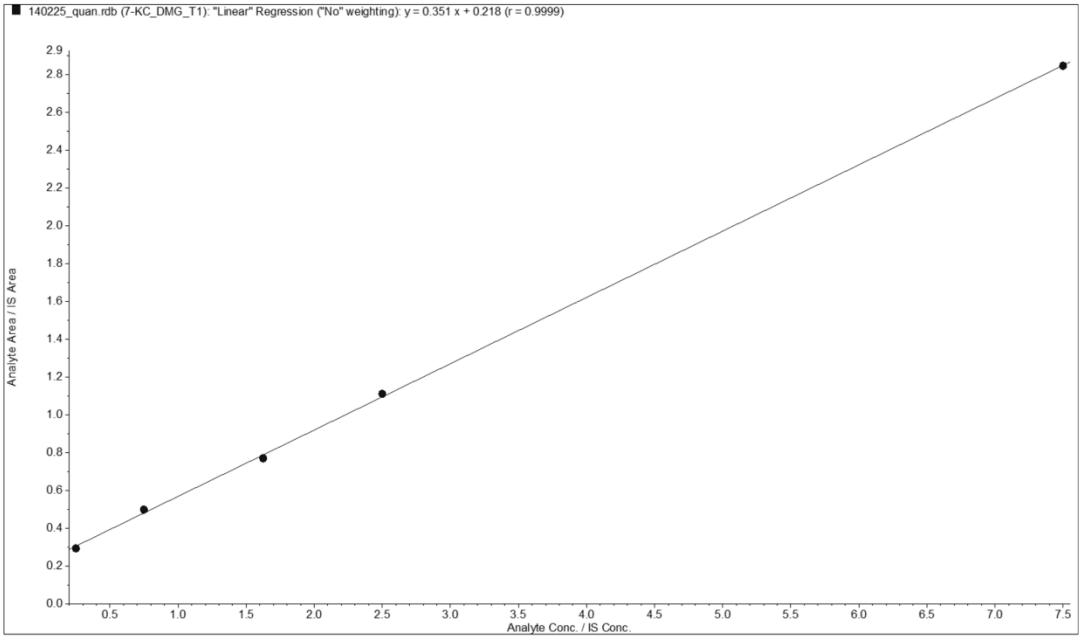
2.1.7 SUPPORTING INFORMATION

Appendix 1: Calibration curve examples

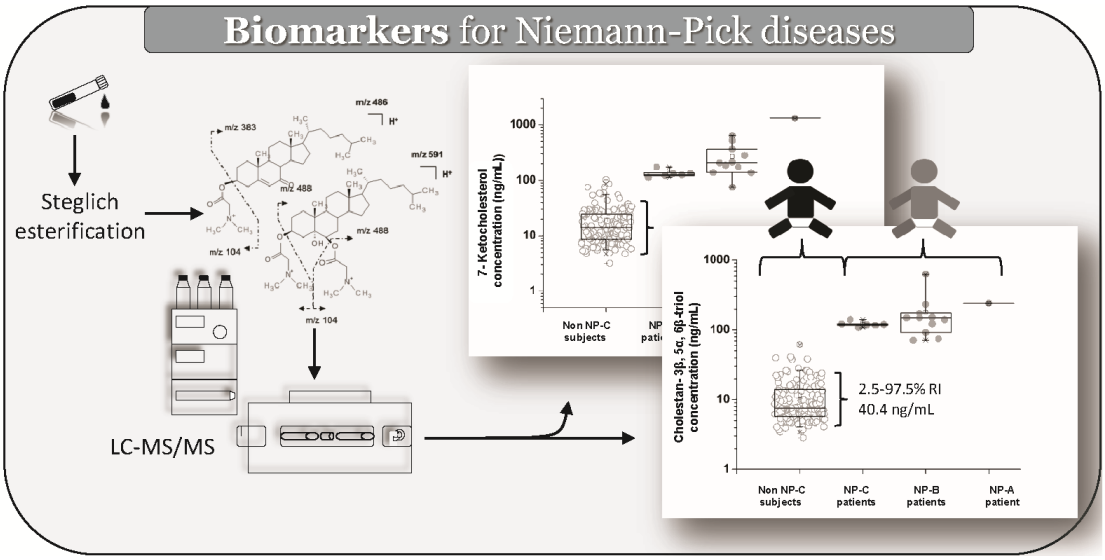
Cholestane-3β,5α,6β -triol



7-Ketocholesterol



Supporting Information Figure 1| Calibration curve examples



Supporting Information Figure 2| Graphical Abstract

2.2 LC-MS/MS METHOD FOR OXYSTEROL ANALYSIS IN THE SCOPE OF INFLAMMATORY BOWEL DISEASES

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2.2.1 ABSTRACT

2.2.4.1 BACKGROUND

Inflammatory bowel diseases (IBDs) are a group of chronic relapsing immune-mediated disorders of the gastrointestinal tract impairing significantly the patient's quality of life with still badly understood pathophysiology. A set of oxysterol receptors have recently been implicated in IBD, namely the Epstein-Barr virus-induced G-protein coupled receptor 2 (EBI2), the Liver X receptors (LXRs) and the RAR-related orphan receptor gamma t (RORγt). We surmised that the oxysterols triggering these receptors may play a role in the pathophysiology of IBD and that there are differences in plasma levels between healthy controls and different IBD forms.

2.2.4.2 METHODS

We established a LC-MS/MS (ESI^+) method to measure 24(S)-hydroxycholesterol (24(S)-OHC), 25-hydroxycholesterol (25-OHC), 27-hydroxycholesterol (27-OHC), 7α,24(S)-dihydroxycholesterol (7α,24(S)-OHC), 7α,25-dihydroxycholesterol (7α,25-OHC), 7β,25-dihydroxycholesterol (7β,25-OHC), 7α,27-dihydroxycholesterol (7α,27-OHC), and 7β,27-dihydroxycholesterol (7β,27-OHC) in human plasma. The method was subsequently applied to determine the oxysterol profile in plasma of healthy volunteers (n=22) and patients with Crohn's disease (CD) (n=20) and Ulcerative colitis (UC) (n=23) during active and inactive disease stage.

2.2.4.3 RESULTS

The reported method is linear ($r > 0.99$), sensitive (detection limit ranging from 0,0018 nM to 0,24 nM for dihydroxycholesterols, and from 0,0077 nM to 1,09 nM for monohydroxycholesterols) and precise, with a median intra-day imprecision of 12 % and 8,8 % and a median inter-day imprecision of 4,5 % and 5,7 % for dihydroxycholesterols and monohydroxycholesterols, respectively. Recoveries for all oxysterols in the inter-day assay ranged between 82 % and 124 %. All oxysterols were separated in one chromatographic run using acetonitrile in the mobile phase. However, to increase oxysterol's ionisation for MS/MS detection, in particular for matrixes containing low oxysterol concentrations, a second mobile phase using methanol instead of acetonitrile was applied. With these methods, 24(S)-OHC, 25-OHC, 27-OHC, 7α,25-OHC and 7α,27-OHC could be detected in human plasma of controls and IBD patients. The levels of 27-OHC and 25-OHC were lower in CD and UC patients compared to the control group, while the level of the other oxysterols did not differ.

2.2.4.4 CONCLUSIONS

With the LC-MS/MS assay reported here we were able to measure 24(S)-OHC, 25-OHC, 27-OHC, 7α,25-OHC and 7α,27-OHC in plasma of healthy controls, CD and UC patients in active and in remission stage. The results indicate that IBD patients have lower 27-OHC and eventually 25-OHC levels in the active and the remission phases compared to controls, indicating a role for the oxysterol-LXR-axis in IBD.

2.2.2 INTRODUCTION

Inflammatory bowel diseases (IBDs) are a group of chronic relapsing immune-mediated disorders of the gastrointestinal tract that are pathologically characterised by intestinal inflammation and epithelial injury [1,2]. Clinical symptoms are various and include tiredness, weight loss, fever, night sweats, decreased appetite, nausea, vomiting, stomach pain, flush, and (bleeding) bowels, and they all strongly impair the patient's quality of life [3,4]. The disease onset typically occurs between 15 and 35 years, although IBD can occur in infants [4–6]. The highest incidence rates and prevalences for IBD are found in industrialised countries and societies with western lifestyle like Europe and North America [7]. In Europe about 2,5–3 million people are estimated to have IBD and despite many research efforts, the exact etiology is still unknown [5,8,9].

IBD's main diseases are Crohn's Disease (CD) and Ulcerative colitis (UC), which share a similar clinical pathological phenotype. The difference between the two diseases lies in the localisation of the inflammation in the gastrointestinal tract, in immunological and histological patterns and in disease-specific complications [10,11].

The particularity of IBDs is the relapsing character with remission periods of various length in which the inflamed mucosa of the gastrointestinal tract comes back to normal [3,11]. Ideally, the aim of IBDs management is to keep the remission stage for as long as possible and to decrease the period and the severity of the disease activity during flare-ups [3,12].

Oxysterols are oxidised derivatives of cholesterol being either of enzymatic or non-enzymatic origin with very few examples deriving from both origins [13,14]. They mainly regulate cholesterol homeostasis, however, recently some enzymatically derived oysterols and their receptors have been implicated in inflammatory diseases [13,15–19].

24-hydroxycholesterol (24(S)-OHC), 25-hydroxycholesterol (25-OHC), and 27-hydroxycholesterol (27-OHC) are ligands for the liver X receptors (LXRs), LXRα and LXRβ, involved in fatty acid and cholesterol metabolism [20–27]. Two studies recently indicated that the oxysterol/LXR axis may play a role in IBD. Polymorphisms in LXRs were shown to be associated with IBD in a Danish study and the mRNA expression for both LXRs are decreased in CD and UC patients compared to controls [28,29].

7β,27-dihydroxycholesterol (7β,27-OHC) and 7α,27-dihydroxycholesterol (7α,27-OHC) bind to the RAR-related orphan receptor gamma t (RORγt), which drives the differentiation of $CD4^+$naïve cells (CD referes here to cluster of differentiation) into pro-inflammatory T helper 17 cells (T_H17 cells) [30]. T_H17 cells are implicated in CD and UC, although their exact role is not clear yet [31–37].

Additionnaly, 7α,27-OHC together with the more potent 7α,25-dihydroxycholesterol (7α,25-OHC),) and 7β,25-dihydroxycholesterol (7β,25-OHC) were discovered to be Epstein-Barr virus-induced G-protein coupled receptor 2 (EBI2) ligands mainly involved in B cell motion in lymphoid tissues [20,21,38,39]. Increased EBI2 expression was detected in the ileum of CD patient with nucleotide-binding oligomerisation domain 2 (NOD2) mutations and a polymorphism in EBI2 showed an association with IBD in a genome-wide association study (GWAS) [40–42].

These findings led us to surmise that enzymatically derived oxysterols binding to the EBI2, LXRs and RORγt receptors **(Figure 1)** may play a role in the pathophysiology of IBD and that there are differences in plasma levels between healthy controls and patients with CD and UC.

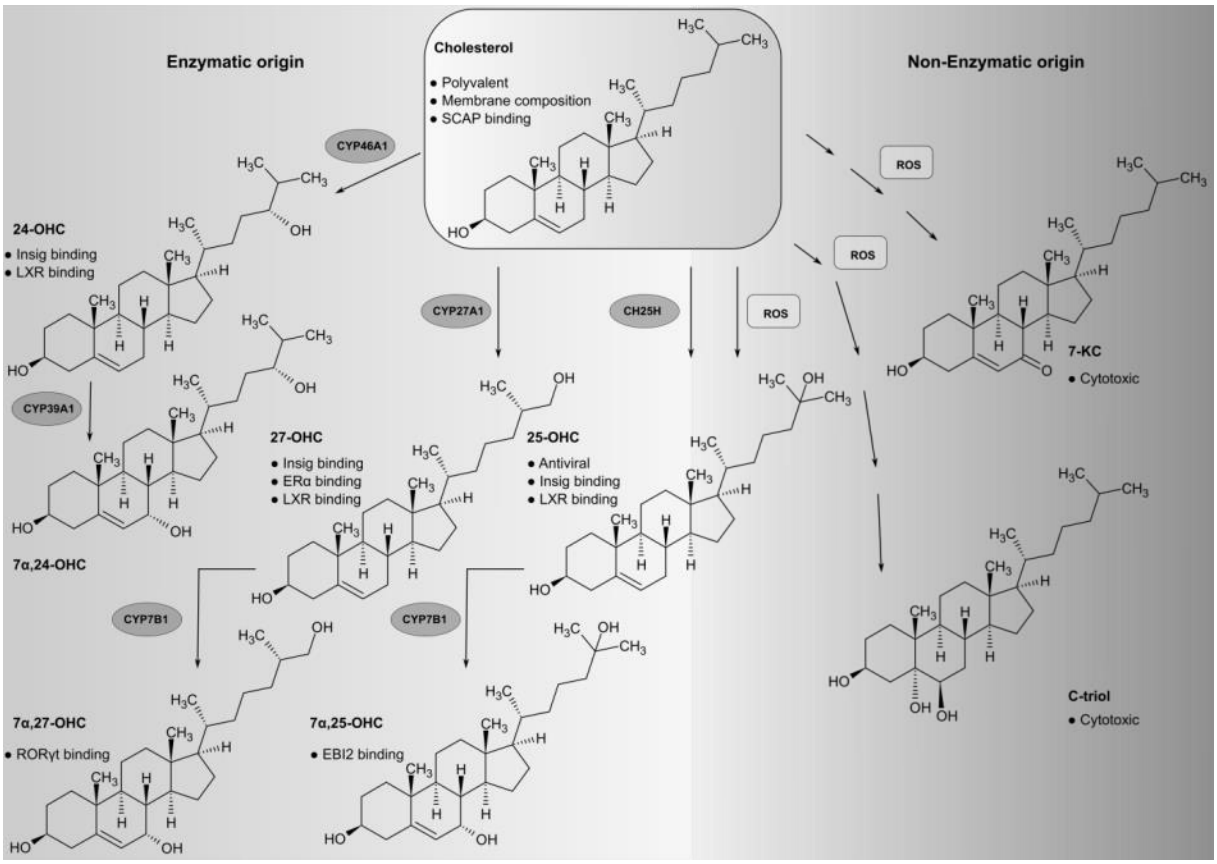

Figure 1| Oxysterols from enzymatic and non-enzymatic origins. Cholesterol is transformed to monohydroxycholesterols as for example 24(S)-hydroxycholesterol (24(S)-OHC), 27-hydroxycholesterol (27-OHC) and 25-hydroxycholesterol (25-OHC) through the enzymatic action of cholesterol 24-hydroxylase (CYP46A1), sterol 27-hydroxylase (CYP27A1) or cholesterol 25-hydroxylase (CH25H), respectively. 24-OHC is then converted to 7α,24(S)-dihydroxycholesterol (7α,24-OHC) by the action of the oxysterol 7α-hydroxylase 2 (CYP39A1), while 27-OHC and 25-OHC are both converted to their respective dihydroxycholesterols by the oxysterol 7α-hydroxylase (CYP7B1). Additionnaly to the enzymatic conversion, 25-OHC derives also from non-enzymatic origin through radical oxygen species (ROS). Other examples of non-enzymatic generated oxysterols are 7-ketocholesterol (7-KC) and cholestane-3β,5α,6β-triol (C-triol). The abbreviations and acronyms are explained in the chapter. Figure adapted from Cyster *et al*.(2014) [15].

2.2.3 MATERIAL AND METHODS

2.2.4.1 CHEMICALS

Oxysterols and their deuterated standards, 7α,24(S)-dihydroxycholesterol with > 99 % purity, 7α,25-dihydroxycholesterol with > 99 % purity, 7β,25-dihydroxycholesterol with > 99 % purity, 7α,27-dihydroxycholesterol with > 99 % purity, 7β,27-dihydroxycholesterol with > 99 % purity, 24(S)-dihydroxycholesterol with > 99 % purity, 25-dihydroxycholesterol with > 99 % purity, and 27-dihydroxycholesterol with > 99 % purity, 26,26,26,27,27,27-[2H6]7α,25-dihydroxycholesterol with > 99 % purity, 25,26,26,27,27,27-[2H6]7α,27-dihydroxycholesterol with > 99 % purity, 25,26,26,27,27,27-[2H6]7β,27-dihydroxycholesterol with > 99 % purity, 25,26,26,26,27,27,27-[2H7]7α,24(R/S)-dihydroxycholesterol with > 99 % purity, 26,26,26,27,27,27-[2H6] 25-dihydroxycholesterol with > 99 % purity, and 25,26,26,27,27,27-[2H6] 27-dihydroxycholesterol with > 99 % purity were bought from Avanti® Polar Lipids, Inc. Alabaster (Ala), USA. Butylated hydroxytoluene (BHT) was purchased from Sigma-Aldrich Chemie GmbH, Buchs, Switzerland. Solvents used for LC mobile phases are: absolute methanol and acetonitrile (ACN), which were obtained from Chemie Brunschwig AG, Basel, Switzerland. Ultrapure water was obtained with the Elga-Purelab Genetic System from Labtech Service, Wohlen, Switzerland. Formic acid (FA) and ethanol (EtOH) were obtained from Sigma-Aldrich Chemie GmbH, Buchs, Switzerland.

2.2.4.2 PREPARATION OF INTERNAL STANDARD AND CALIBRATOR SOLUTIONS

Deuterated internal standards (IS) and BHT were dissolved in ethanol (EtOH) and mixed together to obtain a final concentration of 2 µM and 37,5 nM, respectively, and stored at -80°C until use. A stock solution of 24(S)-OHC, 25-OHC, 27-OHC, 7α,24(S)-OHC, 7α,25-OHC, 7β,25-OHC, 7α,27-OHC,and 7β,27-OHC in EtOH was prepared at a concentration of 4,5 µM each. The final concentrations of the calibration points for the dihydroxycholesterols were 2,5, 6, 15, 40, 80, 120, and 175 nM, while for the monohydroxycholesterols they were 6, 15, 40, 80, 120, and 175 nM.

2.2.4.3 SAMPLE COLLECTION AND PREPARATION

For method validation, a plasma pool was made with anonymised left over plasma samples from routine analysis of the Division of Clinical Chemistry and Biochemistry from the University Children's Hospital Zurich. As control samples immediately frozen anonymised Li-heparin plasma of healthy volunteers from the Swiss Red Cross was used, stored at -80°C for no longer than one week. Longitudinal samples in active and in remission stage from UC and CD patients were obtained from the the Swiss IBD Cohort Study. The samples have been stored at -80°C for no longer than 2 years. The Swiss IBD Cohort Study has approval from the local ethics committee and written informed consent was obtained from all patients.

Samples were prepared similarly to the protocol of Karuna *et al* [43]. In brief, 200 µL of plasma were subjected to protein precipitation after the addition of 20 µL of the 2 µM deuterated IS mix containing BHT by the addition of 230 µL cold EtOH, immediately mixed and stored at 4°C for 15 min. This step was repeated three times, adding 450 µL cold EtOH each time now. In total, 1,6 mL of cold EtOH were given to the 200 µL of plasma in a 2mL Eppendorf tube, resulting in a 1:9 dilution. The mixture was mixed and centrifuged at 4°C for 20 min at 16060 g. The supernatant was transferred into a new tube, vortexed and centrifuged a second time at 4°C for 10 min at 16060 g. The supernatant of the second centrifugation step was transferred into a new tube and dried under nitrogen. Reconstitution was done with 75 µL EtOH containing 0,1% FA and then injected into the LC-MS/MS system.

2.2.4.4 LC-MS/MS ANALYSIS

The LC-MS/MS analysis was performed on a Dionex UltiMate 3000 RS (Thermo Scientific, Olten, Switzerland), using an Acquity UHPLC BEH column (150 mm x 2,1 mm; 1,7 µm particle size and 130 Å pore size) from Waters, Brechbühler AG, Schlieren, Switzerland) and coupled with an AB Sciex 5500 TripleQuad (AB Sciex, Zug, Switzerland).

The following gradient was applied using solvent A (H20: MeOH (95%: 5%); 0.1% FA; pH 3) and B1 (MeOH: ACN (62,5 %: 37,5 %; 0.1 % FA) or B2 (MeOH; 0,1 % FA). Solvent B (1 and 2) increased from 0,2 % to 73 % within 0,5 min, and then to 81,2 % within 8 min. After an additional 8 min, solvent B further increased to 98 % in 3,5 min. Next, solvent B decreased to 0,2 % in 4 min and was kept constant for another 3 min. The flow rate was 400 µL/min and injection was performed via a Performance Optimising Injection Sequence (POIS) with the weak solvent being water and the sample volume 10 µL.

The source parameters of the AB Sciex 5500 TripleQuad were CAD: 6, CUR: 15, GS1: 40; GS2: 70, IS: 4500, TEM: 550. Multiple reaction monitoring (MRM) in positive ion mode was used to identify the oxysterols using their precursor and selected product ions as listed in **Table 1,** although the transitions were not selective for one particular oxysterol. For quantification the transition indicated in **Table 1** as "Quantifier" was used and all ions were monitored with a dwell time of 20 msec.

Table 1| MRM settings for all oxysterols

Analyte in positive ion mode $[M+H]^+$ with ESI	**Type of transition**	**Precursor ion (m/z)**	**Product ion (m/z)**	**DP (V)**	**EP (V)**	**CE (V)**	**CXP (V)**
7α,24(S)-OHC 7α,25-OHC 7β,25-OHC 7α,27-OHC 7β,27-OHC	Qualifier	401.3	383.3	74	10	17	32
7α,24(S)-OHC 7α,25-OHC 7β,25-OHC 7α,27-OHC 7β,27-OHC	Quantifier for 7α,24(S)-OHC, 7α,25-OHC, 7β,25-OHC, 7α,27-OHC, 7β,27-OHC	383.3	159.1	11	10	27	14
7α,24(S)-OHC 7α,25-OHC 7β,25-OHC 7α,27-OHC 7β,27-OHC	Qualifier	383.3	145.1	11	10	31	14
d^6 7α,25-OHC d^6 7α,27-OHC d^6 7β,27-OHC	Quantifier for d^6 7α,27-OHC,	407.3	389.3	110	10	13	32
d^6 7α,25-OHC d^6 7α,27-OHC d^6 7β,27-OHC	Qualifier	407.3	371.4	110	10	17	31
d^6 7α,25-OHC d^6 7α,27-OHC d^6 7β,27-OHC	Qualifier	407.3	159.2	110	10	34	14
d^7 7α,24(S)-OHC	Qualifier	408.3	215.1	80	10	33	18
d^7 7α,24(S)-OHC	Qualifier	408.3	390.3	110	10	13	32

Analyte in positive ion mode $[M+H]^+$ with ESI	Type of transition	Precursor ion (m/z)	Product ion (m/z)	DP (V)	EP (V)	CE (V)	CXP (V)
d^7 7α,24(S)-OHC	Qualifier	408.3	372.4	110	10	17	31
24(S)-OHC 25-OHC 27-OHC	Quantifier for 27-OHC	385.2	161	100	10	25	
24(S)-OHC 25-OHC 27-OHC	Quantifier for 25-OHC and 24(S)-OHC	385.2	147.1	100	10	31	
24(S)-OHC 25-OHC	Qualifier	420.2	367.3	130	10	17	
d^6 25-OHC d^6 27-OHC	Qualifier	426.5	391.3	45	10	12	
d^6 25-OHC d^6 27-OHC	Quantifier for d^6 27-OHC	391.3	159.1	120	10	35	
d^6 25-OHC d^6 27-OHC	Quantifier for d^6 25-OHC	391.3	373.3	120	10	15	

2.2.4.5 LINEARITY, PRECISION AND RECOVERY

The linearity was investigated by a 6 point calibration curve for the monohydroxycholesterols and by a 7 point calibration curve for the dihydroxycholesterols in EtOH. Intra-day (n=4) and inter-day imprecision (n=16) were assessed measuring plasma without any oxysterol addition and plasma spiked with 80 nM oxysterols. The absolute recovery of the oxysterols was calculated from the results of the 80 nM spiked plasma samples. For calculation of the recoveries endogenous levels of oxysterols from the non-oxysterol added plasma sample were subtracted from the spiked plasma samples. The obtained values were then divided by the spiked amount, averaged and multiplied by 100 to obtain a percentage value.

2.2.4.6 LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated as follow [44]:

$$LOD = y_{LQC} + 3.3 * s_{LQC}$$

$$LOQ = y_{LQC} + 10 * s_{LQC}$$

With y_{LQC}= y-intercept of the calibration equation
S_{LQC}= standard deviation of the peak area/IS area ratio of the inter-day non-oxysterol added samples

2.2.4.7 DATA ANALYSIS

Data analysis was made with Analyst 1.6.2 and then exported to Microsoft Excel 2010. Oxysterol quantification in IBD plasma samples were performed with a Signal to Noise ratio (S/N) ≥ 5. The average was taken, when two aliquots were available with the same disease activity from the same patient. Statistics were performed with MedCalc® Version 14.8.1 and Prism 6 for MacOSX. Sample size calculation was made with PS Power and Sample Size Calculations version 2.1.30. The comparison between the oxysterol concentrations between the two IBD diseases in active and in remission stage with the control group was made using the Kruskal–Wallis

test. Comparison between the oxysterols concentration of the two disease states within a disease were calculated with the Wilcoxon signed-rank test. For all statistical tests a p-value < 0,05 was considered significant at a level , while a p-value < 0,01 was considered to be highly significant.

2.2.4 RESULTS

2.2.4.1 OXYSTEROL DETECTION AND SEPARATION

10 µL injection of oxysterol standards in EtOH solution result in broad and overlapping peaks. However, if the oxysterol standard solution is simultaneously injected with a weak solvent (water) before and after the oxysterol standard solution, then the peak signals are thin and the analytes separated. This analyte band compression is called Performance Optimising Injection Sequence (POIS) by Phenomenex ® **(Figure 2)** [45].

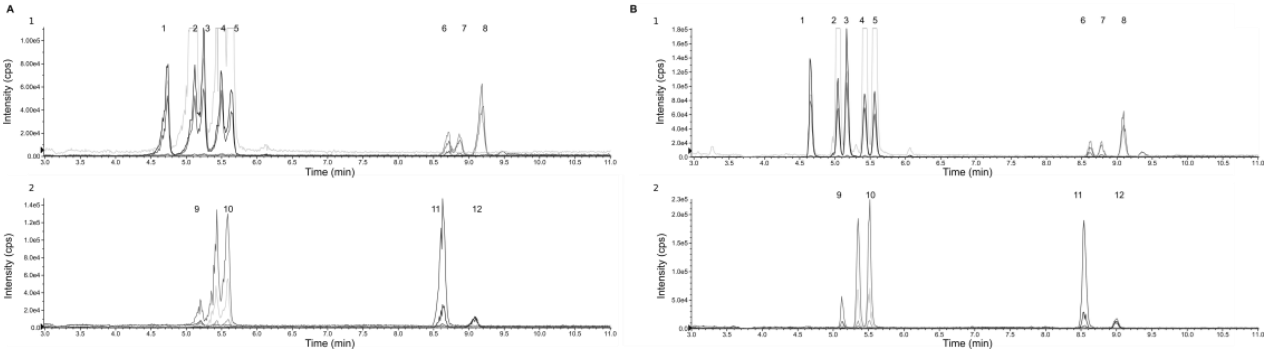

Figure 2| Extracted ion chromatogram (XIC) for oxysterol standards (A1) and deuterated standards (A2) obtained with 10µL regular injection of 120 nM oxysterols in EtOH (A) compared to the XIC for oxysterol standards (B1) and deuterated standards (B2) obtained with 10µL injection with the POIS procedure (B). The oxysterol standards of the chromatogram are 7β,25-OHC (1), 7β,27-OHC (2), 7α,25-OHC (3), 7α,24(S)-OHC (4), 7α,27-OHC (5), 25-OHC (6), 24(S)-OHC (7), and 27-OHC (8). The deuterated standards of the chromatogram are d^6 7α,25-OHC (9), d^6 7α,27-OHC (10), d^6 25-OHC (11), and d^6 27-OHC (12).

Atmospheric pressure chemical ionisation (APCI) is the traditional ionisation technique for underivatised oxysterols, however, recently Karuna *et al.* (2015) reported better oxysterol ionisation with Electrospray (ESI), contradicting so the old dogma [43,46–48]. We tested the oxysterol detection using both ionisation sources and obtained significant better results using ESI, thereby supporting the statement of Karuna *et al*. (2015) **(Supporting Information Figure 5)** [43].

Using POIS and ESI, all oxysterol standards can be separated in one unique run using mobile phase A (H20: MeOH (95 %: 5 %); 0.1 % FA; pH 3) and B1 (MeOH: ACN (62,5 % : 37,5 %); 0.1% FA) **(Figure 3).**

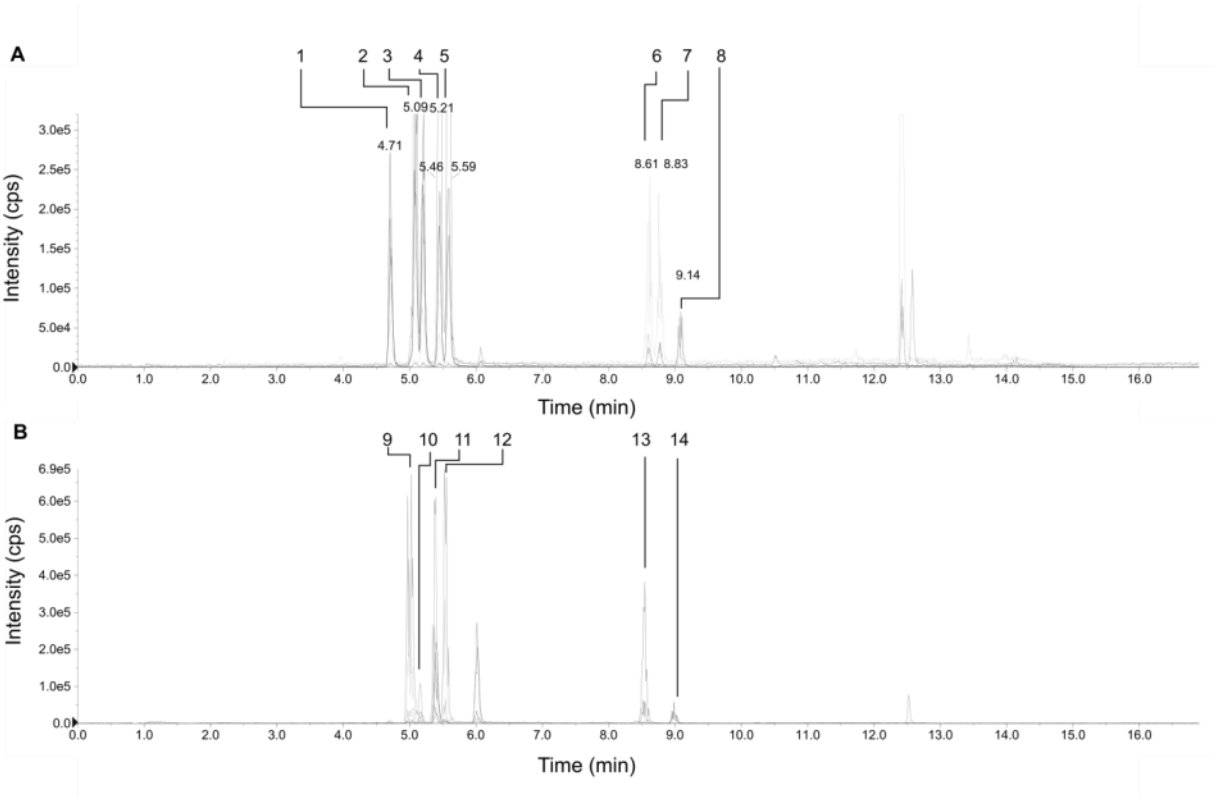

Figure 3| Extracted ion chromatogram of the di- and monohydroxycholesterols. The upper chromatogram (A) represent the di- and monohydroxycholesterol standards with (1) 7B,25-OHC, (2) 7B,27-OHC, (3) 7A,25-OHC, (4) 7A,24(S)-OHC, (5) 7A,27-OHC, (6) 25-OHC, (7) 24(S)-OHC and (8) 27-OHC. The lower chromatogram (B) represent the deuterated di- and monohydroxycholesterols internal standards with (9) d^6 7B,27-OHC, (10) d^6 7A,25-OHC, (11) d^6 7A,24(S)-OHC, (12) d^6 7A,27-OHC, d^6 25-OHC (13), and (14) d^6 27-OHC .

However, with mobile phase B1, we observed decreased oxysterol signal intensity compared to mobile phase B2 (MeOH; 0,1% FA). These observations are in agreement with McDonald *et al*. (2007) who observed a modest to significant decrease in sterol intensity in ACN containing mobile phases [49]. The inconvenience of using mobile phase B2 is that 24(S)-OHC and 25-OHC are co-eluting and cannot be differentiated by mass spectrometrical analysis. We therefore measured samples twice, first with mobile phase A and B1 and then with mobile phase A and B2. All oxysterols with the exception of 24(S)-OHC and 25-OHC were quantified using mobile phase A and B2 **(Table 2).**

Table 2|Peak areas obtained with mobile phase B1 (containing ACN) and with mobile phase B2 (containing MeOH) for all oxysterol standards analysed in this project. Values displayed with * are the sum of 24(S)-OHC and 25-OHC

Analyte	Transitions	Peak area obtained with mobile phase B1 (ACN containing)	Peak area obtained with mobile phase B2 (MeOH containing)	Peak Area obtained with mobile phase B2 / Peak Area obtained with mobile phase B1	Final conclusion for mobile phase B2
24(S)-OHC	385/147	43388	111190*	2,56	↑
24(S)-OHC	385/161	73880	87082*	1,18	↑
25-OHC	385/147	55907			
25-OHC	385/161	587			
27-OHC	385/147	64242	87500	1,36	↑
27-OHC	385/161	106350	161730	1,52	↑
7α,24(S)-OHC	383.3/159	103060	184130	1,79	↑
7α,24(S)-OHC	383.3/145	88183	158950	1,80	↑
7α,25-OHC	383.3/159	224750	314740	1,40	↑
7α,25-OHC	383.3/145	130120	219410	1,69	↑

Analyte	Transitions	Peak area obtained with mobile phase B1 (ACN containing)	Peak area obtained with mobile phase B2 (MeOH containing)	Peak Area obtained with mobile phase B2 / Peak Area obtained with mobile phase B1	Final conclusion for mobile phase B2
7α,27-OHC	383.3/159	107980	173640	1,61	↑
7α,27-OHC	383.3/145	73969	125940	1,70	↑
7β,25-OHC	383.3/159	164290	257470	1,57	↑
7β,25-OHC	383.3/145	95465	190800	2,00	↑
7β,27-OHC	383.3/159	125130	260090	2,08	↑
7β,27-OHC	383.3/145	80907	196990	2,43	↑
d^6 25-OHC	391/159	52606	52606	1,00	-
d^6 25-OHC	391/373	320610	363490	1,13	↑
d^6 27-OHC	391/159	31434	441054	14,03	↑
d^6 27-OHC	391/373	25892	25831	1,00	-
d^6 7α,24(S)-OHC	389/159	41061	62181	1,51	↑
d^6 7α,24(S)-OHC	407/383	320690	315960	0,99	↓
d^6 7α,25-OHC	389/159	136530	214920	1,57	↑
d^6 7α,25-OHC	407/383	166490	217640	1,31	↑
d^6 7α,27-OHC	389/159	50187	75387	1,50	↑
d^6 7α,27-OHC	407/383	314020	4077850	12,99	↑

2.2.4.2 METHOD VALIDATION

The quantification of 24(S)-OHC, 25-OHC, 27-OHC, 7α,24(S)-OHC, 7α,25-OHC, 7β,25-OHC, 7α,27-OHC, and 7β,27-OHC in human plasma was performed by MRM in positive ion mode with the transitions displayed in **Table 1**. The calibration was linear from 6 to 175 nM for monohydroxycholesterols and from 2,5 to 175 nM for the dihydroxycholesterols with a correlation coefficient > 0.99 for all oxysterols. In this assay, the lowest LOD (0,0018 nM) and LOQ (0,0037 nM) was achieved for 7β,25-OHC , while 27-OHC generated the highest LOD (1,09 nM) and LOQ (3,30 nM) **(Table 3)**. Using the method with mobile phase A and B1, the target oxysterols are quantified within 19 minutes with elution times in the range of 4,71 min ± 0,02 min to 5,59 min ± 0,05 min for the dihydroxycholesterols and 8,61 min ± 0,07 min to 9,08 min ± 0,06 min for the monohydroxycholesterols **(Table 3)** and **(Figure 3).** The specificity was ensured by the chromatographic separation of all isomers of the mono- and dihydroxycholesterols and by recording of several transitions per oxysterol group **(Table 1).** The precision of the method was investigated by the intra-day and inter-day imprecision of plasma samples spiked with 80 nM. The median intra-day coefficient of variations (CV) are 12 % (with min 4,5 % and max 13 %) for the dihydroxycholesterols and 8,8 % (with min 1,1 % and max 10,6 %) for the monohydroxycholesterols, respectively. The median inter-day CVs are 4,5 % (with min 4 % and max 8,9 %) for the dihydroxycholesterols and 5,7 % (with min 3,7 % and max 4,9 %) for the monohydroxycholesterols, respectively **(Table 3).** The accuracy of the method was investigated by analysing recoveries of oxysterols in spiked plasma samples. The recoveries varied between 82 % and 124 % for all oxysterols when analysed on four different days **(Table 3)**.

Analyte	Retention time (median ± SD) (min)	R^2	LOD (nM)	LOQ (nM)	Intra-day precision (n=4)			Inter-day precision (n=16)		
					Spiked with oxysterols (nM)	Median (nM) ± SD	CV (%)	Recovery (%)	Median (nM) ± SD	CV (%)	Recovery (%)
7β,25-OHC	4,71 ± 0,02	0,99	0,0018	0,0037	-	-	-	-	-	-	-
					+ 80	97,17 ± 12,61	13	121	97,28 ± 4,35	4,8	124
7β,27-OHC	5,09 ± 0,02	0,99	0,031	0,039	-	-	-	-	-	-	-
					+ 80	67,51 ± 3,21	4,8	95	80,60 ± 3,28	4,1	103
7α,25-OHC	5,20 ± 0,06	0,99	0,0036	0,0057	-	-	-	-	-	-	-
					+ 80	74,46 ± 9,19	12	96	97,92 ± 4,79	4,9	121
7α,24(S)-OHC	5,46 ± 0,04	0,99	0,22	0,49	-	-	-	-	-	-	-
					+ 80	94,16 ± 2,33	2,9	118	98,63 ± 3,95	4,0	120
7α,27-OHC	5,59 ± 0,05	0,99	0,24	0,68	-	-	-	-	-	-	-
					+ 80	73,00 ± 3,31	4,5	91	90,93 ± 3,06	3,7	108
25-OHC	8,61 ± 0,07	0,99	0,0077	0,012	-	-	-	-	-	-	-
					+ 80	71,02± 6,50	9,2	89	75,54 ± 5,48	7,3	96
24(S)-OHC	8,77 ± 0,07	0,99	0,0084	0,022	-	6,54 ± 0,69	10,6	-	6,51 ± 0,37	5,7	
					+ 80	79,83 ± 3,38	4,2	92	74,46 ± 3,28	4,4	82
27-OHC	9,08 ± 0,06	0,99	1,09	3,30	-	66,25 ± 5,85	8,8	-	59,74 ± 5,30	8,9	
					+ 80	145,37 ± 1,59	1,1	99	138,63 ± 5,58	4,0	99
Median monohydroxycholesterols							8,8			5,7	
Median dihydroxycholesterols							12			4,5	

Table 3| Linearity, limit of detection in plasma, limit of quantification in plasma, intra- and interday precision, and recoveries for all oxysterols analysed in this project

2.2.4.3 OXYSTEROL PROFILE IN CROHN DISEASE AND ULCERATIVE COLITIS PATIENTS DURING ACTIVE AND INACTIVE DISEASE PHASE

IBD plasma samples from the Swiss IBD Cohort Study registry and plasma samples of control subjects from the Swiss Red Cross were measured for their oxysterol profile. Generally, the oxysterol concentrations are low, in particular for the dihydroxycholesterols and not all oxysterols were detected **(Figure 4)**. The detected oxysterols, although not in all samples, were 24(S)-OHC, 25-OHC, 27-OHC, 7α,27-OHC and 7α,25-OHC. The monohydroxycholesterols 27-OHC and 24(S)-OHC were detected in all control samples, while they were only present in 88 % and 70 % of UC and CD samples, respectively. In contrast, 25-OHC levels were only detectable in 36 % of control plasma samples **(Supporting Information Table 4).**

The median concentration for 27-OHC in the control group is 79,42 nM, while in the IBD patient samples the 27-OHC concentration is 44,60 nM in active CD patients and 23,36 nM in active UC patients **(Figure 4)**. The 27-OHC concentration in patients in remission is 35,09 nM and 24,76 nM for for CD and UC, respectively. These data show a significant difference of 27-OHC levels between the control group and the IBD patients (p-value = 0,000001) but no significant difference between CD and UC patients.

25-OHC was detected in 36 % of the control samples while no 25-OHC was detected in the CD and UC samples (p-value of 0,0031).

In contrast, no difference was observed for the 24(S)-OHC levels, mainly due to the large spread of values in the different IBD groups. The median 24(S)-OHC was similar between controls (46,42 nM), CD patients (45,41 nM) and UC patients (30,86 nM) with active disease, and in patients in remission (in CD 42,93 nM and in UC 29,37, respectively) **.**

The results are less clear for the dihydroxycholesterols where 7α,27-OHC and 7α,25-OHC were only detected in some controls and IBD samples **(Supporting Information Table 4)**. The fact that only a few samples contained dihydroxycholesterols makes the interpretation very delicate. 7α,25-OHC for instance was rarely but mainly found in the condition of disease remission for both diseases with median concentrations of 7α,25-OHC containing sample of 1,78 nM and 0,70 nM for CD and UC, respectively. Patients in the remission stage with detectable 7α,25-OHC concentration always had measurable 7α,27-OHC, while the opposite was not necessarily true. However, 7α,27-OHC was inconsistently detected in all groups, meaning the control group, CD active, CD in remission, UC active and UC in remission.

To exclude inter-individual differences for the association of the oysterols with disease activity, we compared the oxysterol levels between the active and remission stage of the same CD and UC patients. In doing so, we circumvented oxysterol dependence towards gender, age and ethnicity which was highlighted by Stiles *et al.* 2014 [50]. However, no significant difference was observed between the active and the remission phases of UC or CD patients **(Supporting Information Figure 6).**

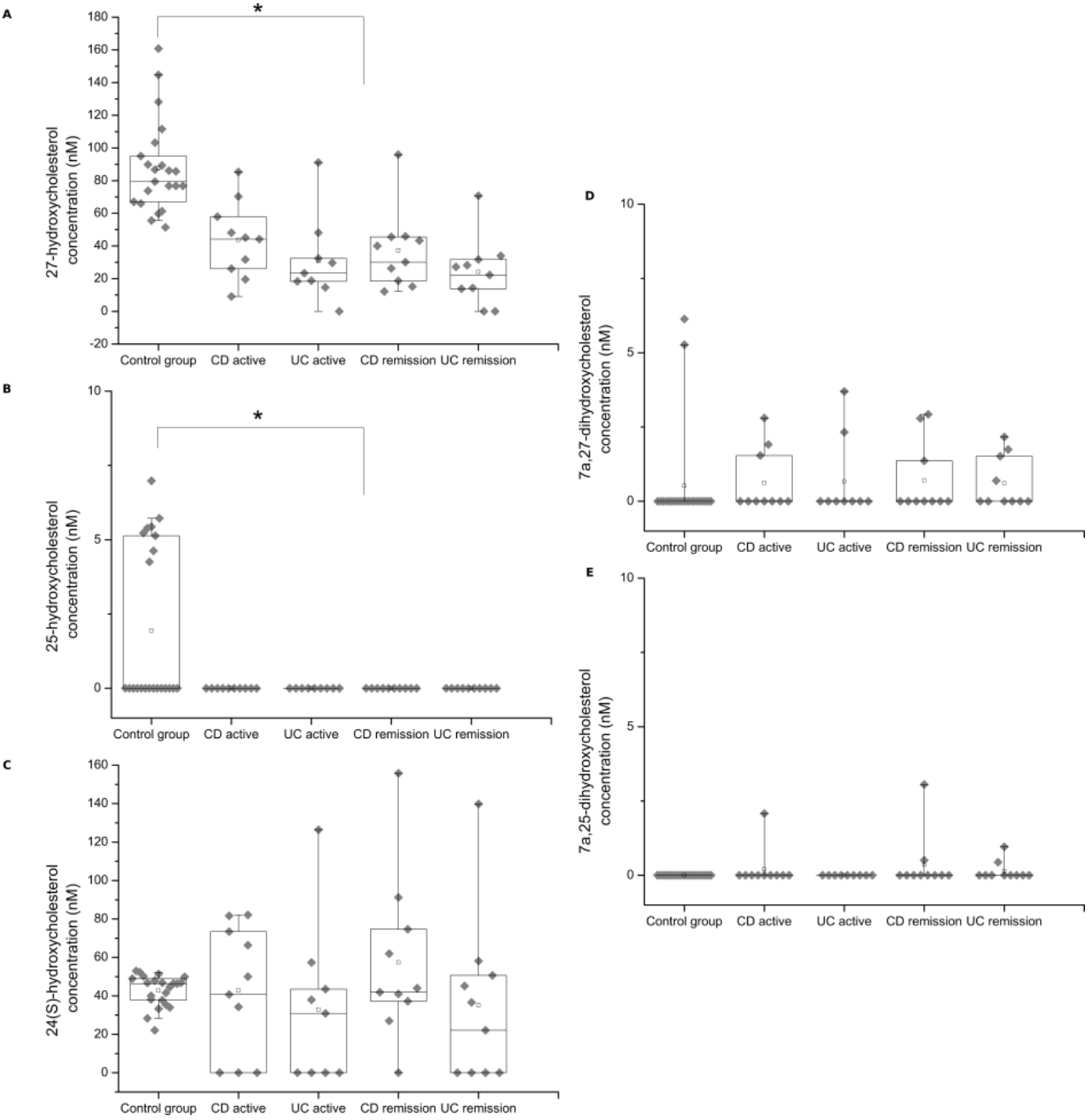

Figure 4| Box- and-whisker plot and raw data representation of 27-OHC (A), 25-OHC (B), 24(S)-OHC (C), 7α,27-OHC (D) and 7α,25-OHC (E) concentration in the control group and in CD and UC patients in active and in remission stage. Box 25th to 75th percentile, line: median, square: mean, box whiskers: 5th and 95th percentiles, asterisk: statistically significant difference.

2.2.5 DISCUSSION

Here, we present a method for the analysis of mono- and dihydroxycholesterols in human plasma in the context of IBD research. The assay validation showed good recoveries (92 % to 125 %) and good precision with median CVs between 4 % and 12 %. In addition, the method is linear over the entire calibration range.

Ideally for analytics, all target analytes are separated by one selective method to avoid confounding factors and misinterpretation. With the aim of separating all oxysterols in one run, we modified the mobile phase composition and gradient of Karuna *et al.* (2015) [43]. Separation of all oxysterols was successfully achieved for the first time, but to do so we used ACN as a mobile phase (B1). Under such conditions the oxysterol signal intensity was decreased compared with mobile phases containing no ACN [49]. The decreased oxysterol signal is not a problem *per se*, as long as the expected signals are high enough for detection. However, oxysterol concentrations in plasma are relatively low and we reached the detection limit for several of the oxysterols in the profile, asking for another mobile phase (B2) using methanol as a solvent. Using this second mobile phase, we were not able to separate 24(S)-OHC from 25-OHC, forcing us to apply the method twice, once with the mobile phase B2 in order to obtain maximal oxysterol signal intensity and a second time with mobile phase B1 to separate and quantify 24(S)-OHC and 25-OHC [43,51–54]. This analysis is a good example in which selectivity can compromise sensitivity and *vice versa*. It should be pointed out that the dihydroxycholesterols as well as the monohydroxycholesterols are isobaric compounds and consequently the precursor ions as well as the product ions are not selective for one specific analyte. The method's selectivity is therefore principally based on the separation obtained by liquid chromatography.

Low analyte concentrations, like oxysterol concentrations in plasma require improved sensitivity. The later is generally obtained with the addition of ionisation-friendly molecules to the target analytes through derivatisation which will lead to enhanced ionisation [55–57]. However, derivatisation of the target analytes has several drawbacks, including the requirement of quantitative and reproducible reaction recoveries in the biological matrix, the requirement to obtain only one derivatisation product for each target analyte and the more laborious sample preparation often including a cleaning step to eliminate the derivatisation reagents. We tested the derivatisation with N,N-Dimethylglycine (data not shown) but we obtained in addition to single derivatised products, twice and three times derivatised products, making quantification difficult and unreliable. In addition, not all oxysterol-dimethylglycine esters could be separated from each other, even with long chromatographic runs. This is why we chose the analysis of underivatised di- and monohydroxycholesterols. Another possible option to improve sensitivity is the choice of the best ionisation source [43,46–48]. AB Sciex 5500 TripleQuad has two possible ionisation sources: APCI and ESI. We compared both ionisation sources and obtained the best results with the ESI sources similar to Karuna *et al.* (2015) [43]. A third possible option to improve sensitivity is to analyse free and esterified oysterols instead of only measuring free oxysterols. Oxysterols are particular in that they are stored in the cell as oxysterol esters but they are only biologically active when they are free [19,22,24,58]. We chose the analysis of free oxysterols to measure only oxysterols potentially acting on their corresponding receptors. However, considering the sensitivity issue, an alkaline hydrolysis step may release the oxysterols from the oxysterol esters, which are then detectable in LC-MS/MS and thus increases the amount of detected oxysterols.

The pre-analytical steps of an analytical method are analyte-dependent and as important as the analysis itself, since they strongly influence the final results. It is therefore difficult to define a general pre-analytical protocol for samples potentially used for several analyses. Oxysterols are not stable more than two years in plasma and only very little stability data are available for oxysterols in plasma [59]. Accordingly, samples for this study were selected from the Swiss IBD Cohort study to have been collected less than two years before analysis. Nevertheless, we observed a trend towards lower oxysterol concentrations in samples for which the time between the blood collection and the biobank storage (delta time) was longer (4 days as maximum). During the delta time, these samples were stored between 2 and 8 °C, which might have an impact on the oxysterol

concentrations. Pre-analytical differences may also explain the large spread of 24(S)-OHC levels in the samples from the Swiss IBD Cohort study, compared to the optimally collected samples from controls.

With this method we were able to measure 24(S)-OHC, 25-OHC, 27-OHC, 7α,27-OHC and 7α,25-OHC in samples of CD and UC patients. The levels are relatively low especially for the dihydroxycholesterols but were found to be in the same order of magnitude as in the literature [43,51–54].

The two monohydroxycholesterols, 27-OHC and 25-OHC, showed both lower levels in IBD patients than in controls, while 24(S)-OHC showed inconclusive results because of the very large concentration range in the IBD patients. 27-OHC is a LXR agonist and Jakobssen *et al.* (2014) observed lower LXRs mRNA levels in CD and UC patients and concluded that triggering LXRs may be protective in IBD [28]. Our data of reduced 27-OHC level in IBD patients would support this notion, indicating that the LXRs as well as their agonist, 27-OHC, are reduced in IBD. However, it is not clear on whether the lowered level of LXRs will lead to reduced oxysterol production, or whether these are two independent events hampering the 27-OHC-LXRs axis. Interestingly, no difference could be found between 27-OHC concentrations in CD and UC patients, indicating a common disease mechanism in both IBD diseases due to inflammation depending on the 27-OHC-LXRs axis. This would also be supported by the data from Jakobssen *et al.* (2014), who also detected similar LXRs mRNA expression levels between CD and UC patients [28].

We detected 25-OHC in 36 % of our control group samples while none of the IBD samples showed detectable levels. The median 25-OHC concentration of 5,39 nM of the 25-OHC containing control samples is in agreement with the values reported in literature [53,54]. Considering these low levels of free 25-OHC in controls, we would anticipate that IBD samples would be under our detection limit, when we consider a similar reduction of 25-OHC in IBD patients as observed for 27-OHC. In order to measure 25-OHC in IBD patients and to eventually make a distinction between controls and IBD patients, an alkaline hydrolysis could be helpful, since 25-OHC is known to be esterified up to 98,7 % [43,50]. It is tempting to speculate that a reduced 25-OHC concentrations in IBD patients could be associated with a reduced anti-viral immune response in IBD patients [15,20,21,60,61].

Since we obtained the most promising results for the LXRs agonists it would be interesting to add further LXRs agonists to the oxysterol panel that are for example not direct dihydroxycholesterol precursors. This would give first indications on whether the LXRs functions are hampered in general in IBD or not. Many LXRs agonists have been reported in in the literature including 7α-hydroxycholesterol (7α-OHC), 4β-hydroxycholesterol (4β-OHC), 22(R)-hydroxycholesterol, 20(S)-hydroxycholesterol, 24(S),25-epoxycholesterol (24(S),25-EC), and cholestenoic acid, a metabolite of 27-OHC [23,24,62,63]. These oxysterols have *in vitro* approximately the same binding affinity to LXRα and LXRβ and thus seem to select the receptor depending on the origin of the oxysterol and on the expression of the receptor. For instance, 24-OHC is only produced in the brain, which expresses also LXRβ forming the 24-OH- LXRβ axis [64–67]. However, the more analytes are measured in a profile, the less sensitive the method will be. Therefore, a compromise needs to be found between the number of analytes to target and the sensitivity to reach.

7α,25-OHC, and 7α,27-OHC were the only detected dihydroxycholesterols. However, the sensitivity of our method did not allow measuring the levels of the dihydroxycholesterols in most plasma samples, limiting the interpretation on their implication in IBD. The few 7α,25-OHC concentrations which were measurable, were slightly higher as the ones reported in the literature (median concentration of 7α,25-OHC containing sample: 1,78 nM for CD in remission and 0,70 nM for UC in remission compared to 0,21 nM from Karuna et *al.* (2015), < 0,48 nM for Griffiths et *al*. 2013, and 0,29 nM for Griffiths et *al*. 2013) [43,53,54]. Interstingly, of the few measurable dihydroxycholesterol levels, 7α,25-OHC containing plasma samples always contained also 7α,27-OHC, eventually indicating an activation of the oxysterol-EBI2-axis in IBD. To corroborate such an association we would need further improvement of the sensitivity of the method. An alkaline hydrolysis could be performed to measure the esterified oxysterols in addition to the free. Both dihydroxycholesterols were reported to be esterified by 73.8 % and 82.0 %, allowing the improvement of a factor of seven to eight for the detection limit [43].

The other three dihydroxycholesterols 7α,24(S)-OHC, 7β,27-OHC and 7β,25-OHC where not detected in our study. It is possible that they either do not exist *in vivo* (as 7β,25-OHC) or that they are not present in measurable amounts in the human body circulation (likely for 7α,24(S)-OHC and 7β,27-OHC) [30,43,76,77]. We come to these statements since to our knowledge no cholesterol β-hydroxylase was shown to exist endogenously questioning the *in vivo* synthesis of both 7β-dihydroxycholesterols. Usually the 7β-dihydroxycholesterols have been included in oxysterol profile analyses to ensure the correct identification of 7α,25-OHC [43]. However, Soroosh *et al.* were able to measure 3-12 nM 7β,27-OHC in mouse spleen suggesting that at least one of the two 7β-dihydroxycholesterols really exists *in vivo* [30]. In contrast, 7α,24(S)-OHC was shown to occur in liver and the enzymes required for its synthesis are well characterised. However, this dihydroxycholesterol seems to be intracellularly localised because no 7α,24(S)-OHC concentrations have ever been reported in plasma [76,77]. Hence human plasma concentrations of these three dihydroxycholesterols are below the current detection limit, which agrees well with the literature.

In conclusion, we show that IBD patients have lower 27-OHC and eventuallay 25-OHC levels in the active and the remission phases compared to controls, indicating a role for the oxysterol-LXR-axis in IBD.

2.2.6 REFERENCES

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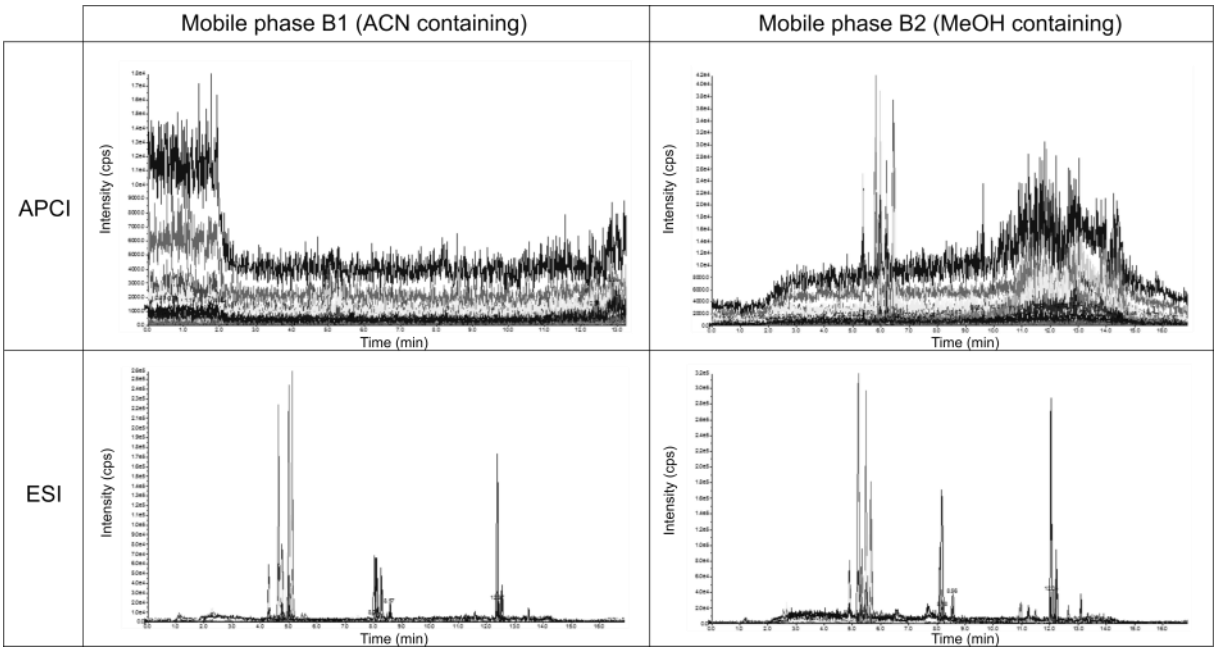
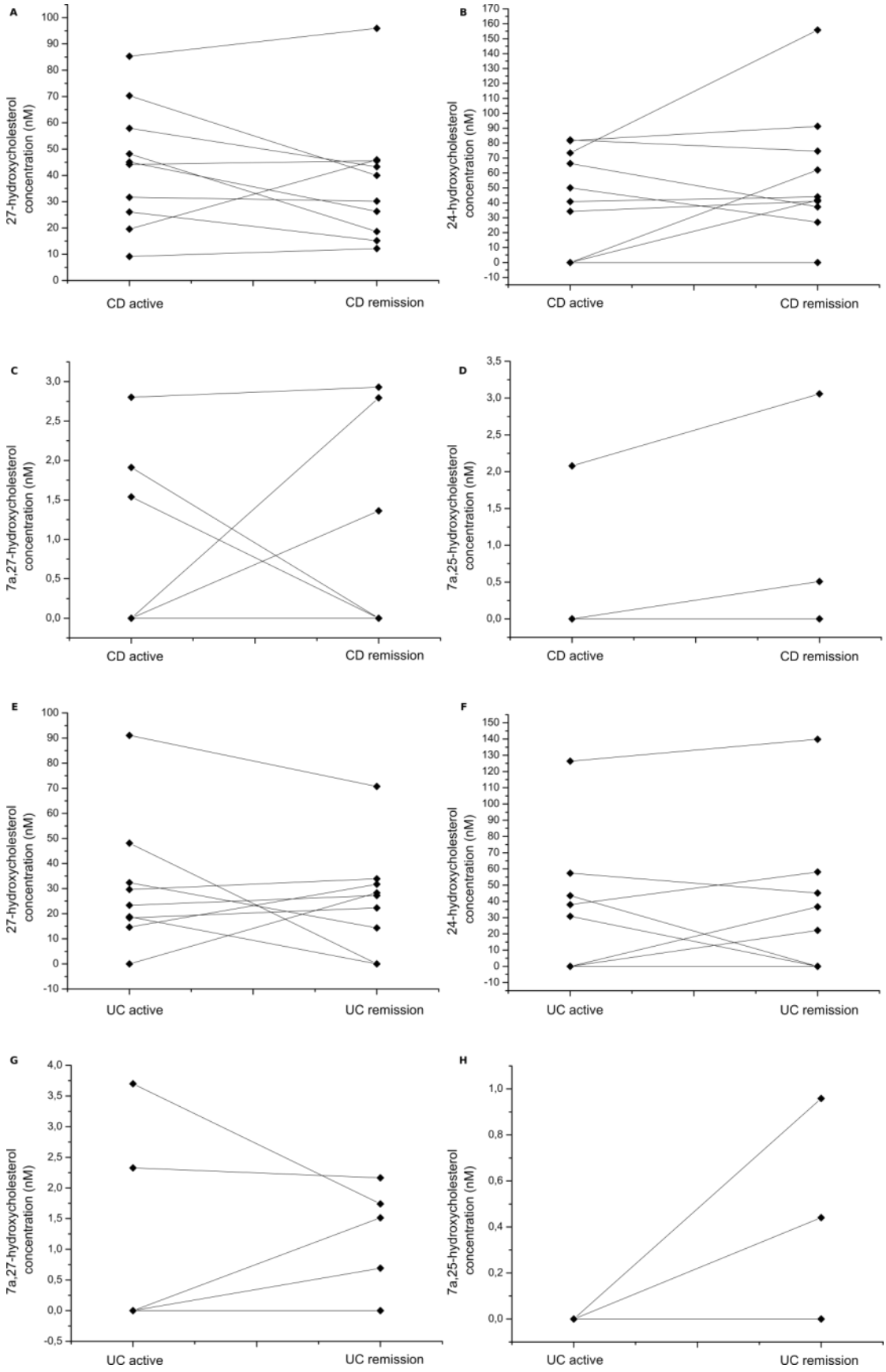
2.2.7 SUPPORTING INFORMATION



Supporting Information Figure 5| Comparison between oxysterol ionisation with APCI and ESI ionisation sources using mobile phase B1 (ACN containing) and mobile phase B2 (MeOH containing).

Supporting Information Table 4| Table summarising the results obtained for 24(S)-OHC, 27-OHC, 7α,27-OHC and 7α,25-OHC in CD and UC patients in active and in remission stage. For each condition the number of hits, the calculated median of that condition, the total number of samples of that condition, and the percentage of hits of that condition were given. Values with * indicates that the median was made out of all oxysterol containing samples instead of the total number of samples.

Condition	27-OHC	25-OHC	24(S)-OHC	7a,27-OHC	7a,25-OHC
Positive counts	60	8	52	14	5
Total samples	65	65	65	65	65
Positive counts %	92 %	12 %	80 %	22 %	8 %
Median concentration in CD and UC (nM)	29,65	0,00	40,76	0,00	0,00
Standard deviation in CD and UC (nM)	23,82	0,00	38,75	1,05	0,57
Upper range in CD and UC (nM)	95,93	0,00	155,78	3,70	3,06
Lower range in CD and UC (nM)	0,00	0,00	0,00	0,00	0,00
Control hits	22	8	22	2	0
Median Control (nM)	79,42	5,39*	46,42	5,70*	0
Control n_{total}=	22	22	22	22	22
Control (%)	100 %	36 %	100 %	9 %	0 %
CD active hits	10	0	7	3	1
Median CD active (nM)	44,60	0	45,41	1,91*	2,08*
CD active n_{total}=	10	10	10	10	10
CD active (%)	100 %	0	70 %	30 %	10 %
CD remission hits	10	0	9	3	2
Median CD remission (nM)	35,09	0	42,93	3,01*	0,00*
CD remission n_{total}=	10	10	10	10	10
CD remission (%)	100 %	0 %	90 %	30 %	20 %
UC active hits	8	0	5	2	0
Median UC active (nM)	23,36	0	30,86	2,79*	1,78*
UC active n_{total}=	9	9	9	9	9
UC active (%)	89 %	0 %	56 %	22 %	0 %
UC remission hits	10	0	9	4	2
Median UC remission (nM)	24,76	0	29,37	1,63*	0,70*
UC remission n_{total}=	14	14	14	14	14
UC remission (%)	71 %	0 %	64 %	29 %	14 %



Supporting Information Figure 6| Line array representation for 27-OHC (A and E), 24(S)-OHC (B and F), 7α,27-OHC (C and G) and 7α,25-OHC (D and H) in active and remission stage for each patient in CD (A-D) and UC (E-H).

3. CONCLUDING REMARKS

3.1 GENERAL CONSIDERATIONS ON THE ANALYSIS OF OXYSTEROLS

Traditional sample preparation methods for oxysterol analysis were optimised to take into account the possible *in situ* oxidation of cholesterol or of oxysterols themselves resulting in an array of different oxysterols. The fear was to obtain erroneous results not reflecting the *in vivo* oxysterol signature. Antioxidants -mainly BHT- were therefore added to the matrix directly after collection and the samples were immediately stored at -80°C. This approach of direct antioxidant addition is ideal but could be avoided with the use of a faster, simpler and gentler sample preparation for LC-MS/MS compared to the sample preparation for GC-MS as demonstrated by Helmschrodt *et al*. (2014) [1]. In the particular case of 4β-OHC, an oxysterol of both enzymatic and non-enzymatic origin, it was shown that the addition of BHT had no influence on the finally measured concentrations and that if oxidation occurred, it was negligible [2]. Similarly, we showed that C-triol and 7-KC are stable in plasma at room temperature for four days, indicating that oxidation does not occur immediately [3]. This point is crucial when working with matrices like plasma obtained from external clinicians or collaboration partners. It is very difficult or even impossible to obtain a reliable antioxidant addition to the sample from clinicians or external collaboration partners. We conclude that accurate pre-analytics for the analysis of C-triol and 7-KC is possible also without antioxidant addition like BHT to the plasma.

Oxysterol research is strongly dependent on selective and sensitive analytical systems. Further development is still required to obtain more insides into the role of oxysterols in human physiological and pathophysiological functions. The oxysterol research still needs to find answers to the many open questions as for example the question of the oxysterol implication in inflammatory disorders such as IBD, besides our finding of lowered 27-OHC and 25-OHC levels. From the chromatography point of view many improvements of conventional HPLC systems were achieved in the last 15 years with the most success for UHPLC. The appearance of commercial sub-2 mm columns used in UHPLC gave a new sparkle on liquid chromatography. UHPLC provides enhanced chromatographic resolution resulting in more narrow peaks with consequently less compounds co-eluting and the detection of approximately 20 % more compounds [4,5]. Additionally, the more narrow peaks of UHPLC give a better signal to noise ratio than conventional HPLC, thus increasing the sensitivity and the reproducibility of the analysis [6]. The higher velocity applied in UHPLC results in shorter analysis times increasing the sample throughput by a factor of 9 while maintaining similar efficiency to HPLC [7]. Other advantages are the diversity of columns regarding stationary phases which are not only restricted to reverse phases and commercially available column lengths [8]. Existing HPLC methods can be easily transferred to UHPLC methods without the need of extra qualified staff [7]. The major but also the only drawback of this technology is the generated high background pressure requiring special and costly instrumentation [8]. A conceivable near future chromatography advance with a potential for acceptance by the oxysterol community is the concept of ultra-performance convergence chromatography (UPC^2). This technology has not yet been tested for oxysterols, but Waters demonstrated in an application note the efficient and short separation of several steroids with UPC [2] [9]. The concept of ultra-performance convergence chromatography (UPC^2) was first mentioned by Giddings in 1964, but the first commercially available easy-to-handle system was introduced by Waters (ACQUITY UPC^2 ™) only at the Pittcon conference in 2012. UPC^2 is based on the control of density, diffusivity and viscosity of a supercritical fluid-based mobile phase, generally CO_2. For increased selectivity, diverse stationary phases are available and any organic solvent can be added as a co-solvent, if deemed necessary [10]. According to Gopaliya *et al*. this technology combines the advantages of LC and GC with increased analytes diffusion into the mobile phase giving a better peak separation, possible analysis of thermo-sensitive substances not resistant to high GC temperatures, and the low viscosity of supercritical fluids resulting in higher flow speed compared to HPLC [11].

From the point of view of mass spectrometry, high resolution mass spectrometers (HRMS) are the most promising instruments with already some established oxysterol methods [12–15]. High resolution mass spectrometers have the advantage of high mass resolving power and mass accuracy making the distinction possible between almost isotopic peaks [16]. However, until a few years ago high resolution was only possible with Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometers which have a very limited scan speed [17]. High scan speed was possible to achieve with time-of-flight (TOF) mass spectrometers but these

instruments do not reach the same resolving power and mass accuracy as FT-ICR does [18–20]. Hybrid orbitrap instruments are the third type of recent analytical instruments attaining high resolution (QExactive from Thermo Fisher Scientific: 140 000 at 200 m/z). Their advantages are numerous, including high mass accuracy (QExactive < 5ppm), high scan speed (QExactive: 12 scans/sec), fast polarity switching and a large dynamic range (QExactive: three order of magnitude) [21]. From a practical point of view the hybrid orbitrap instruments are benchtop instruments and don't require expensive cooling apparatuses to maintain a superconducting magnet as it is the case for FT-ICR MS [22]. From an experimental point of view, these types of instruments have the benefit to allow untargeted data collection for identification of unexpected compounds, retrospective review of the data and in case of untargeted experiments a simpler method development since they don't require compound-specific sample preparation. However, high resolution mass spectrometers are expensive instruments and data analysis can be very complex. An important drawback for quantification is that the sensitivity does not reach these achieved by triple quadrupoles yet [23]. We are convinced that the improvement of sensitivity is just a matter of time and will be soon equivalent to the triple quadrupoles allowing high specificity and sensitivity.

An important issue that still deserves attention is the fact that oxysterol MS analysis is hampered in two ways by the chemical structure of the oxysterols. The first issue is the poor ionisation of oxysterols due to their apolar nature and their lack of ionisation-friendly chemical groups containing for example oxygen or nitrogen atoms [23]. The general rule for ESI is: the more polar groups a compound possesses the better it can be ionised. In the case of monohydroxycholesterols the ionisation can only take place at the oxygen at the C-3 position of the sterol ring and at the oxygen present on the side chain. Dihydroxycholesterols for instance already ionise more readily than monohydroxycholesterols since they possess three possible ionisation sites. Positive ion formation in ESI can be slightly enhanced through the addition of traces of formic acid, usually at 0,1% v/v [24]. The second issue of oxysterol MS analysis is the inefficient fragmentation due to the stability of the steroid backbone. During fragmentation the basic steroid structure is maintained with only the different substituents being separated, forming mainly one or two neutral water molecules. Even further fragmentations (MS^2 and more) at higher energies give very little structural information [25]. The unspecific oxysterol fragments don't allow to distinguish between isobaric oxysterols, making the identification impossible without a good separation by liquid chromatography [26].

3.2 CONCLUDING REMARKS ON OXYSTEROLS AS BIOMARKER FOR NP-C DIAGNOSIS

Rare diseases in Europe are defined by Orphanet as "diseases which affect a small number of people compared to the general population and specific issues are raised in relation to their rarity. In Europe, a disease is considered to be rare when it affects 1 person per 2000" [27]. One rare disease example is NP-C with an estimated occurrence of 1 per 120.000 live births [28]. NP-C disease onset, symptoms, and progression differ strongly among patients hindering thereby a straightforward diagnosis [29]. The complex and often delayed diagnosis probably overlooks especially patients with milder forms which may become symptomatic in adulthood. Thus the real incidence of the disease is possibly higher than estimated. The lack of a fast, sensitive and specific diagnostic assay for NP-C was till recently an additional burden for NP-C diagnosis. It was therefore a big turmoil when Porter *et al.* discovered two biomarkers for NP-C [30,31]. It is in this context that the University Children's Hospital Zurich being one of the rare medical centers with NP-C diagnostic expertise, decided to implement a method for identification and quantification of the reported biomarkers. The aim was to diagnose NP-C in a screening-like manner in suspected cases. This method was successfully achieved as part of this thesis and since January 2015 the Division of Clinical Chemistry offers NP-C diagnosis on a fortnight basis. In the meantime, other laboratories in Europe offer a NP-C diagnostic assay based on oxysterol detection such as the laboratory of Münster (D), Manchester (UK), Padova (I), and Udine (I), thus covering different European countries for NP-C diagnosis in a very short time span. The NP-C diagnostic assay implementation by several groups has the advantage of inter-lab method comparison several times a year, guaranteeing accurate analytics for patient safety. However, with the necessary hindsight's and the attention of many professional from different fields, several criticisms have emerged to which we would like respond.

The first and major issue concerns the biomarker specificity. C-triol and 7-KC are products of oxidative stress and are not of enzymatic origin. The provenance of the oxidative stress in NP-C cells is currently unknown, but probably the excessive cholesterol accumulation in the lysosomes is responsible for cellular dysfunctions leading to oxidative stress. However, oxidative stress is not specific to NP-C and is known to be involved in several other diseases as for example Parkinson's disease, Atherosclerosis, Diabetes or Asperger syndrome [32–35]. Indeed in the meantime, C-triol and 7-KC were found to be also elevated in other diseases such as, NP-A, NP-B, lysosomal acid lipase deficiency or CTX, thus reducing their specificity for NP-C [3,36]. In addition to false positive results, several groups reported false negative results during the 2014 Oxysterol Workshop in Münster. Based on these two issues, A. Dardis proposed at the same workshop to add the oxysterol assay as screening tool at the first position of the NP-C diagnostic algorithm but with no intention to make it a standalone method, and especially not replacing any former diagnostic methods. This illustrates the fact that every diagnostic method has some limitations regarding its sensitivity (ideally, 100 % detection of the true positive values) and its specificity (ideally, to have no false positive values). The most important aspect is to be aware of it. This oxysterol assay proposed here constitutes an important step ahead towards fast diagnosis of new patients, which is of inestimable value for the affected patients and families. Since we started with this assay in Switzerland, we were already able to diagnose 5 new cases, for a disease occurring only in 1 of 120.000 births.

The second issue concerns the collection of reference values. Reference values are an important tool to distinguish healthy subjects from patients with a particular disease and can be derived by three different methods. The first method consists in calculating the mean ± standard deviation of the values obtained from a healthy population. However, this is only possible with a Gaussian distribution of the values, or a non-Gaussian distribution transformed to a Gaussian after logarithmic plotting [37]. The second and most widespread method is the determination of the 2,5 - 97,5 percentile for a healthy population after eventually removing extreme outliers[37]. The last method being relatively uncommon is the application of the second method on a "not really healthy" population such as hospitalised infants in contrary to a healthy population [37]. To do so has two advantages: first in the context of reference ranges for children it is extremely difficult to obtain sufficient healthy donors with an informed consent between 1 day and 18 years old. Second, the assay still needs to be sufficiently discriminative between the "not really healthy" population and patients because this is the regular clientele for laboratory analyses in the case of NP-C suspicion. In those cases the 2,5 - 97,5 percentile obtained

from a “not really healthy” population is much more robust for discrimination than the one obtained from healthy subjects [37].

The third issue concerns sample preparation itself. Traditional oxysterol research methods contain an oxysterol-cholesterol separation step *via* SPE [38,39]. This procedure is applied to avoid interference by cholesterol present in a concentration that is three orders of magnitude higher than the oxysterols. However, nowadays selective MRM scanning for the target analytes are applied, making the cholesterol separation step unnecessary. A second argument for the cholesterol separation step is the possible *in situ* oxidation of cholesterol during sample preparation. However, we showed that both oxysterols are stable in plasma for up to four days at room temperature indicating that if any oxidation occurs, it remains negligible.

In addition to the three criticisms discussed above, it is worth noting that a novel derivatisation reagent for the oxysterol assay in the NP-C context has been described, while we were implementing the oxysterol assay [40]. The sample preparation is very similar to the derivatisation with N,N-dimethylglycine but much faster. However, this derivatisation reagent is not commercially available and needs to be synthesised regularly since it is only stable for 1 week. Even if the sample preparation itself is faster than our method, we doubt that the proposed method would find acceptance in clinical laboratories since it requires regular preparation of the derivatisation reagent hampering stat-analysis of urgent samples.

3.3 CONCLUDING REMARKS ON THE ROLE OF OXYSTEROLS IN IBD AND THEIR ANALYSIS

3.3.1 THE ROLE OF OXYSTEROLS IN IBD

The analysis of mono-and dihydroxycholesterols as biomarkers of IBD is a novel approach which shows some promises. The most important finding in this context is that 27-OHC and eventually 25-OHC are reduced in IBD patients compared to healthy volunteers. Unfortunately the data obtained for 24(S)-OHC being the third LXRs agonist of this study, are inconclusive and cannot be used for interpretation. Since 27-OHC and 25-OHC are LXRs agonists, our findings could be an indication on impaired LXRs activity in IBD. However, it is still unclear which monohydroxycholesterol of these three activates the most strongly the LXRs *in vivo* in IBD. It was shown that *in vitro* the binding force to LXRs is in the following descending order: 24(S)-OHC > 25-OHC ≥ 27-OHC, indicating that 27-OHC is not the most important monohydroxycholesterol LXRs agonist [41–43]. Consequently it would mean that the reduced 27-OHC concentrations in IBD would not have much effect on the LXRs activity. However, it is uncertain to which extend this *in vitro* findings are relevant in the *in vivo* situation. It is to denote that, in human plasma the monohydroxycholesterol concentrations are in the decreasing order: 27-OHC > 24(S)-OHC > 25-OHC, placing 27-OHC as the first possible LXRs agonist. Furthermore Fu *et al.* demonstrate that the 27-OHC concentration was dose dependent of the cholesterol loading in human monocyte-derived macrophages and that 27-OHC activates LXRs actually in a dose-dependent matter [44]. In addition, they showed that 27-OHC is an agonist of both LXRα and LXRβ [44]. Taking all the data together it appears that the *in vivo* situation is unclear but that 27-OHC is still a possible important LXRs agonist in IBD.

Setting up a panel of analytes for a method asks to define the number of analytes necessary to most likely answer the research question. We covered all EBI2 ligands, since we were particularly interested in their implication in IBD. In addition we included the RORγt ligand that is supposed to have opposite effects to the EBI2 ligands. Additionally we included the precursors of the dihydroxycholesterols - LXR ligands, for which we paradoxical obtained the most promising results. Since we obtained the most promising results for the LXRs agonists it would be interesting to add further LXRs agonists to the oxysterol panel that are for example not direct dihydroxycholesterol precursors. This would give first indications on whether the LXRs functions are hampered in general in IBD or not. Many LXRs agonists have been reported in in the literature including 7α-OHC, 4β-OHC, 22(R)-hydroxycholesterol, 20(S)-hydroxycholesterol, 24(S),25-EC, and cholestenoic acid, a metabolite of 27-OHC [41–43,45]. These agonists have *in vitro* approximately the same binding affinity to LXRα and LXRβ and thus seem to select the receptor depending on the origin of the oxysterol and on the expression of the receptor. For instance, 24-OHC is only produced in the brain, which expresses also LXRβ forming the 24-OH-LXRβ axis [46–49]. However, the more analytes are measured in a profile, the less sensitive the method will be. Therefore, a compromise needs to be found between the number of analytes to target and the sensitivity to reach.

Additionally to the possible expansion of the oxysterol panel for LXRs agonist, it would be interesting to deeper study the influence of the suppression of LXRs activity in IBD on downstream target gene expression. LXRs are master transcriptional regulators controlling sterol and fatty acid metabolism through up-regulating genes implicated in fatty-acid synthesis (SREBP-1c, and fatty acid synthase (FAS)), in cholesterol clearance (cholesterol 7α-hydroxylase, and apoE), cholesterol cell efflux (ABCA1, ABCG1, ABCG5, and ABCG8), and acting on the metabolism of plasma lipoproteins (cholesteryl ester transfer protein, and lipoprotein lipase (LPL)) [41,50–56]. In addition they negatively regulate cholesterol biosynthesis through regulation of the lanosterol 14α-demethylase (CYP51A1) and squalene synthase [57]. It could be helpful to unravel if LXRs agonist diminution in IBD influences one particular, several or all of these target genes. It would help to better understand the inflammatory pathways occurring in IBD as well as possible cell protective mechanism when LXRs activation is inappropriate.

The observation of reduced LXRs agonists in IBD redirected our interest for the implication of LXRs in inflammation, which appeared in the last years to be of protective nature. An important discovery going in the direction of LXRs protective role was the finding of the anti-inflammatory and anti-viral properties of 25-OHC already mentioned earlier [58–63]. In brief, Type 1 IFNs release is an important host defence mechanism towards bacterial or viral TLRs ligands that increases CH25H expression and consequently 25-OHC synthesis [64–66]. 25-OHC activates LXRs which effects are mutually antagonistic to the action of SREBP2 being thereby suppressed and in turn decreases the expression of IL-1β and the inflammasome activity [58,59,66]. In addition, reduced SREBP2 activity decreases the sterol biosynthesis and thus contributes to decrease virus cell entry and viral replication [67]. Furthermore it was shown that the addition of synthetic LXR agonist to LPS stimulated macrophages, inhibited their expression of pro-inflammatory mediators [68,69]. This discovery was taken as explanation for the reduced inflammation observed in murine models of contact dermatitis and atherosclerosis after administration of synthetic LXR ligands [68,70,71]. Our results motivated us to further research in literature for eventually other discoveries not mentioned yet which would support the protective action of LXRs in immunity. Indeed, Joseph *et al.* discovered decreased macrophages resistance to apoptosis when challenged with *Listeria monocytogenes* in LXRs null macrophages [72]. SPα is a soluble protein of the scavenger receptor cysteine-rich superfamily expressed by macrophages in lymphoid tissue with antiapoptotic functions [72,73]. The authors could show that LXRs directly activate SPα and thereby limiting the apoptosis of macrophages during bacterial infection and consequently increases the antimicrobial activity of macrophages [72]. Additionally a second discovery was descripted being in the same context as the first one. It reported that apoptotic cell engulfment activated LXRs in mice and that LXRs subsequently induced the expression of Mer, which is a tyrosine kinase crucial for phagocytosis [74]. This pathway of Mer activation by LXRs was shown to occur in macrophages as well as in neutrophils and is important for appropriated apoptotic cell clearance and maintenance of the immune tolerance in mice [74,75]. Taking both discoveries together, LXRs showed indeed to have a protective function during inflammation in increasing the macrophage resistance and increasing the phagocytic activity of immune cells. From this it appears that LXRs have direct immune functions but it is not elucidated to which extend the LXRs induced suppression of the cholesterol biosynthesis and the increased fatty acid metabolism mentioned earlier is involved in the protective LXRs immune functions. It is for instance known that isoprenoid being early-stage intermediate of the cholesterol biosynthesis pathway are important for protein prenylation [76]. Protein prenylation occurs among other on proteins involved in inflammatory responses such as the farnesylation on Ras (Rat sarcoma protein) or the geranylgeranlyation on Rac1 (Ras-related C3 botulinum toxin substrate 1) [76,77]. All the data converge to an important contribution of LXRs in inflammation, although it is at the moment not known which of them is the most important in IBD.

In order to obtain first hints on oxysterol signature in plasma of IBD patients we actually conducted a small "pre-study" with samples of 24 IBD patients of the Division of Gastroenterology and Hepatology of the University Hospital Zurich. In two of these samples we observed an additional peak which is not one of our analytes. The chromatographic behaviour of this compound is to elute between the IS of 7α,27-OHC and 7α,24-OHC in the ACN gradient, while with the MeOH gradient this compound elutes between 7α,24-OHC and 7α,25-OHC, having the same transitions as all other dihydroxycholesterols. We found out that both samples belonged to women with UC with strong disease activity and we were excited about the relevance of this additional peak in UC. However, in the Swiss IBD cohort study we detected only one sample with this additional peak and the sample belonged to a man with CD in remission stage. The inconsistence between the different samples with the additional peak indicates that it might be an interference with no relevance for the IBD research.

3.3.2 ANALYSIS OF OXYSTEROLS IN THE SCOPE OF IBD

Generally sample preparation of plasma is relatively simple and the availability of plasma is good compared to other biological tissues. This makes plasma the first-choice matrix for method development. We also followed this strategy, although plasma is most likely not the most oxysterol-rich tissue of the human body, since the oxysterol synthesising enzymes are located for instance in the liver, in lymphoid tissues, in the small intestine, in the brain, in the lung or in the kidney [78,79]. In order to obtain a more comprehensive picture of the role of

oxysterols in IBD we would like to analyse oxysterols in other tissues, in particular in intestinal tissue left over from colectomy. In parallel to the oxysterol determination in humans, we would also like to analyse them in mice in particular in the lymph nodes where the EBI2 receptor is expressed, in the spleen and in the liver. Oxysterol extraction from mouse liver is already being implemented in our laboratory, while this manuscript was in preparation.

As already highlighted throughout this manuscript, several techniques to improve the LC-MS/MS sensitivity for oxysterol analysis have been described. In addition to these general improvements, in the context of the oxysterol method for IBD research we encountered the specific problem of relatively high background level for the monohydroxycholesterol transitions even for oxysterol standards dissolved in EtOH. Testing different EtOH qualities for the standards did not improve this issue. Since our ACN and MeOH mobile phase solvents were of very high purity, we suppose that the water quality of the mobile phase was insufficient for this type of analysis. Karuna *et al.* (2015) used for their oxysterol method water of high purity, confirming the importance of the solvent's purity degree for the method [80]. However, buying extra pure water increases the running costs of the analysis.

3.4 CONCLUSION

In this work, two LC-MS/MS methods were established for the analysis of oxysterols in human diseases. The first method was developed and implemented for the analysis of oxysterols of non-enzymatic origin in a clinical setting of NP-C. In addition, a second method was optimised for the analysis of enzymatically-derived oxysterols in the plasma of IBD patients, opening a new research domain. Even if these molecules have the reputation of being challenging to analyse, in both projects, a sensitive, selective and robust assay was essential for the detection, identification and quantification of the targeted oxysterols. In the first project, we tested the approach of oxysterol derivatisation, while in the second project we analysed underivatised oxysterols. In both projects dealing with two very distinct human diseases, oxysterols were evaluated for their use as biomarkers. For the NP-C diagnostic we conclude that they effectively fulfil the function of biomarker, although with no absolute disease specificity. On the contrary, the discovery of the oxysterol signature in IBD is in its beginning and it is too early to make a statement. However, the results are very promising for the monohydroxycholesterols to serve as novel biomarker for IBD.

3.5 REFERENCES

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29-30.10.2014	Swiss Society of Clinical Chemistry (SGKC), Congress Centre Basel, Basel, Switzerland.
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09.04.2015	14th Clinical Research Day, University Hospital Zurich, Switzerland
22.06.2014	13th International Congress of Paediatric Laboratory Medicine, Istanbul, Turkey.
23.10.2014 **25.10.2013** **25.10.2012**	4th, 3rd and 2nd Retreat of the Children's Research Centre, Tagungszentrum Schloss Au, Wädenswil, Switzerland.
29.08.2014 **23.08.2013**	10th and 9th Symposium of the Zurich Centre for Integrative Human Physiology, Zurich, Switzerland.

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